

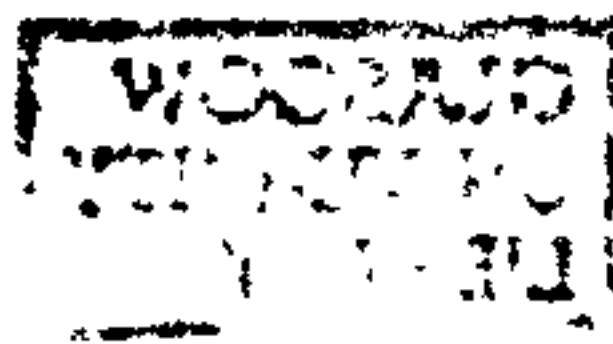
Equine Interferon-gamma and Associated Cytokines

By

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Declaration

The studies described in this thesis were performed in the Department of Veterinary Pathology, University of Glasgow Veterinary School, and are of the exclusive responsibility of the author except for the following procedures. Collection of post-mortem samples performed by Mr. Richard Irvine (Post-Mortem room, Glasgow University Veterinary School). Production of equine interferon-gamma synthetic peptides, performed by Mr Tom Dunsford (Department of Veterinary Pathology, University of Glasgow Veterinary School). Inoculation of rabbits with equine interferon-gamma derived synthetic peptides and recombinant equine interferon-gamma, described in chapter 3, performed, respectively, by Dr. David Argyle (Department of Veterinary Clinical Studies, University of Glasgow Veterinary School) and Dr. Jay Patel (Intervet UK, The Elms, Cambridge). Flow cytometry, described in chapter 3, performed by Dr. Linda Andrew (Leukemia Research Fund Unit, Department of Veterinary Pathology, University of Glasgow Veterinary School). Production of DNA vaccination constructs and execution of the vaccination trial, described and acknowledged in chapters 4 and 6, respectively.

Mauro Nuno Pereira Gonçalves



Abstract

Cytokines are small proteins or glycoproteins that mediate cellular growth and differentiation and regulate immune responses. Upon encounter with antigen, CD4⁺ T cells are able to influence the character of the immune response elicited through the expression of distinct types of cytokines. Th1 cytokines, especially IFN- γ but also TNF- β and IL-2, constitute one such pattern of expression, promoting cell mediated immune responses. In a broader sense, interleukin-12 and interleukin-18 can also be classified as type I cytokines in as much as they are able to shift the CD4⁺ cytokine expression pattern to a Th1 phenotype and specifically stimulate IFN- γ production not only by T helper cells, but also by cytotoxic T cells and natural killer cells.

The purpose of the present project was to develop equine Th1 cytokines, making them available for dissecting the equine immune system, particularly in what concerns to defence mechanisms against infectious micro-organisms. Following the trend in human medicine, the cytokines produced will be useful for the development of new therapeutics and prophylactics to be used in the control and prevention of infectious diseases of the horse. For this effect we have initiated studies on equine interferon-gamma and related cytokines and obtained the following results. Equine interferon-gamma was cloned and produced in a variety of heterologous protein expression systems. The biological activity of recombinant equine interferon-gamma was assessed *in vitro*. Polyclonal serum preparations against equine interferon-gamma, obtained by immunization with recombinant protein, were recovered and characterised. Also described is the cloning of the interferon-gamma inducing cytokines equine interleukin-12 and equine interleukin-18. The potential use of the cloned equine cytokine genes as vaccine adjuvants was evaluated by DNA co-administration with plasmids encoding the equine influenza virus antigens haemagglutinin and nucleoprotein, followed by viral challenge.

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Abbreviations

GENERAL ABBREVIATIONS

°C	degrees celsius
µl	microlitre(s)
µM	micromolar
A	adenine or adenosine
A _{260/280/600}	absorbance at 260, 280 or 600 nm
Ab	antibody
ABTS	2-2'-azinobis(3-ethylbenzothiazoline)-6-sulfonic (acid)
AcMNPV	Autographa californica multiple nuclear polyhedrosis virus
AEBSF	4-2-(aminoethyl)-benzenesulfonyl fluoride
Ag	antigen
AP	alkaline phosphatase
APS	ammonium persulphate
bp	base pair
Bq	Becquerel
BSA	bovine serum albumin
C	cytosine or cytidine
CD	cluster of differentiation
cDNA	complimentary deoxyribonucleic acid
CFA	complete Freund's adjuvant
Ci	curie
CMV	cytomegalovirus
ConA	concanavalin A
CPE	cytopathic effect
cpm	counts per minute
Da	dalton
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle medium
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
EEK	equine embryonic kidney
ELISA	enzyme linked immunosorbent assay
eq	equine
ER	endoplasmic reticulum
EtBr	ethidium bromide
FACS	fluorescence assisted cell sorter (or sorting)
FBS/FCS	fetal bovine serum/fetal calf serum
FITC	fluorescein isothiocyanate

FPLC	fast protein, peptide or polynucleotide liquid chromatography
G	gauge; guanine or guanosine
g	gravity; gram(s)
GAP	GTPase activating protein
GCG	Genetics Computer Group
GST	glutathione S-transferase
HBSS	Hank's buffered saline solution
HBV	hepatitis B virus
HEPES	<i>N</i> -2-hydroxyethylpiperazine- <i>N'</i> -2-ethanesulphonic acid
HIV	human immunodeficiency virus
HPLC	high profile liquid chromatography
HRP	horse radish peroxidase
IFA	incomplete Freund's adjuvant
Ig	immunoglobulin
i.d.	intradermal
i.m.	intramuscular
IPTG	isopropyl-1-thio- β -D-galactoside
IU	international unit
kb	kilobase
kDa	kilodalton
KoAc	potassium acetate
L	litre
LB	Luria Bertani medium
LPS	lypopolysaccharide
M	molar
MAP	multiple antigenic peptide; mitogen-associated kinase
MDBK	Madin-Darby bovine kidney
MEM	minimum essential medium
mg	milligram(s)
mM	micromolar
M-MuLV	Moloney murine leukaemia virus
mRNA	messenger ribonucleic acid
NEAA	non essential amino acids
nm	nanometre
oligo(dT)	oligodeoxythymidylic acid
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
pfu	plaque forming units
PHA	phytohaemagglutinin
PMA	phorbol myristate acetate
PMSF	phenylmethylsulphonyl fluoride
poly(A)	polyadenylated (mRNA)

RBC	red blood cell
RIA	radioimmunoassay
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
RPMI	Rosewell Park Memorial Institute
rRNA	ribosomal ribonucleic acid
RSV	Roux sarcoma virus
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
T	thymine or thymidine
TBE	Tris/borate/EDTA
TBS-T	tris buffered saline-tween solution
TCID	tissue culture infectious dose
TE	Tris-EDTA buffer
TEA	Tris/EDTA/acetate
TEMED	<i>N,N,N',N'</i> - tetramethyl-ethylenediamine
T _m	melting (or midpoint) temperature
Tris	tris(hydroxymethyl)aminomethane
Tris-HCl	Tris hydrochloride
UV	ultraviolet
UWGCG	University of Wisconsin Genetics Computer Group
VSV	vesicular stomatitis virus
WHO	World Health Organisation
Xgal	5-bromo-4-chloro-3-indolyl- β -D-galactoside

CYTOKINES

BDNF	brain-derived neurotrophic factor
CLMF	cytotoxic lymphocyte maturation factor (IL-12)
CNTF	ciliary neurotrophic factor
CSF	colony stimulating factor
CSF-1	colony stimulating factor-1 (M-CSF)
EGF	epidermal growth factor
EPO	erythropoietin
EqIFN	recombinant equine interferon
EqIL	recombinant equine interleukin
G-CSF	granulocyte colony stimulating factor
GGF	glial growth factor
GH	growth hormone
GM-CSF	granulocyte macrophage colony stimulating factor
HGF	hepatocyte growth factor
IFN	interferon
IGF	insulin like growth factor
IGIF	interferon gamma inducing factor (IL-18)

IL	interleukin
M-CSF	macrophage colony stimulating factor
MCP	macrophage chemoattractant protein
NGF	nerve growth factor
NKSF	NK stimulatory factor (IL-12)
PDGF	platelet derived growth factor
PRL	prolactin
SCF	stem cell factor
SCF-R	stem cell factor receptor
R	receptor (cytokine receptor)
TGF	transforming growth factor
TNF	tumour necrosis factor
VEGF	vascular endothelial growth factor

AMINO ACIDS

Amino acid	Three letter code	One letter code	Solubility
Aspartic acid	Asp	D	hydrophilic
Glutamic acid	Glu	E	
Asparagine	Asn	N	
Glutamine	Gln	Q	
Lysine	Lys	K	
Arginine	Arg	R	
Histidine	His	H	
Glycine	Gly	G	
Alanine	Ala	A	
Cysteine	Cys	C	
Serine	Ser	S	
Threonine	Thr	T	
Proline	Pro	P	
Tryptophan	Trp	W	hydrophobic
Tyrosine	Tyr	Y	
Valine	Val	V	
Methionine	Met	M	
Isoleucine	Ile	I	
Leucine	Leu	L	hydrophobic
Phenylalanine	Phé	F	

CHAPTER 1

GENERAL INTRODUCTION

1 Introduction

The main objectives of this project were the expression and investigation of the biological activity *in vitro* of equine interferon gamma and an assessment of the potential application of equine interferon gamma and other Th1 type cytokines as viral vaccine adjuvants. This chapter comprises a brief overview of cytokines followed by a focus on the biology of interferon gamma. The description of procedures used for the study of equine interferon gamma will be addressed in chapter 3. Additional work on the cloning of alternative equine cytokines, cloning and expression of equine influenza virus antigens to be used in a vaccine trial and the design of the trial itself will be approached in chapters 4, 5 and 6, respectively, and pertinent background information will be introduced in the individual chapters. Other work, including the cloning of equine transforming growth factor beta-1, and interleukin-1 β and of porcine interleukin-18 will not be mentioned.

1.1 Properties of cytokines

The term cytokine is loosely applied to intercellular polypeptide messengers including interleukins, lymphokines, monokines, interferons, colony stimulation factors and non-haematopoietic growth factors (Nicola, 1994). Due to the striking number of novel cytokines reported in recent years and to the variability that they show at both structural and functional levels, any definition that attempts to cover this heterogeneous group of molecules is necessarily ambiguous. Cytokines can however be defined as secreted regulatory proteins which bind to specific receptors on target cells exerting multiple effects including modulation of immune and inflammatory responses. The pleiotropic activities of cytokines comprise the survival, growth, differentiation and/or specialised effector functions of target cells (Nicola, 1994; Vilcek, 1998). Some characteristic features (Vilcek, 1998; Pugh-Humphreys and Thomson, 1998) that can be attributed to most cytokines are as follows:

- Cytokines are simple proteins or glycoproteins with a molecular weight commonly less than 30 kDa;

- Although some constitutive expression of cytokines has been observed, they are usually tightly regulated at transcriptional or translational level;
- The notion that cytokines act locally (in an autocrine or paracrine fashion) is supported by observations that they are expressed transiently and have a short half life in circulation. Recent data suggests that cytokines can be directionally secreted, therefore targeting cells closely associated with the producing cell;
- Cytokines are produced and active at extremely low (nanomolar to picomolar) concentrations, promoting their actions through binding to specific, high-affinity, cell surface receptors;
- Cytokines act by altering the pattern of gene expression of target cells, leading to stimulation or inhibition of cell proliferation, differentiation and effector functions.

Because some of these features also apply to polypeptide hormones it is important to make a distinction between this two groups of molecules (table 1.1).

Table 1.1 Differential features of polypeptide hormones and cytokines (Vilcek, 1998).

Hormones		Cytokines	
Feature	Exceptions	Feature	Exceptions
Produced by one type of specialised cells	-	Produced by more than one type of cell	IL-2, IL-3, IL-4, IL-5, produced by lymphoid cells only
Each hormone is unique in its actions	-	Different cytokines have overlapping activities	-
Restricted target cell specificity and limited spectrum of actions	Insulin	Multiple target cells and multiple actions (pleiotropy)	-
Act at a distant site (endocrine activity)	-	Short action radius (autocrine or paracrine)	Many (e.g. TGF- β , TNF- α , IL-6)

Due to the multiplicity of cytokines, potential target cells and to the variety of effects exerted, care should be taken in attributing specific activities to individual cytokines. The activation status of the cells (determined by antigen, costimulatory and adhesion

molecules) and the cell cycle stage play an important role in the differential response to cytokines. Additionally, the fact that cytokines do not act alone but instead as components of a cytokine network with synergistic, antagonistic, overlapping and transmodulating activities, leads to the notion of redundancy in cytokine activity.

1.2 Classification of cytokines

Table 1.2 Classification of cytokines (modified from Nicola, 1994).

Cytokine class	Subgroup	Receptor class	Examples	Bio-activities
4- α helical bundle	Short chain	Hemopoietin domain (γ -subunit)	IL-2, IL-4, IL-7, IL-9, IL-13	T cell, B cell, and M ϕ proliferation and differentiation
		β -subunit	IL-3, IL-5, GM-CSF	Neutrophil, Eosinophil, M ϕ and Mast cell proliferation and differentiation
		Ig-TK	M-CSF, SCF	M ϕ and stem cell proliferation and differentiation
		IFNR	IFN- γ	Immune regulation, antiviral activity
	Long chain	Hemopoietin domain (gp130-subunit)	IL-6, IL-11, CNTF	Pleiotropic in liver, nerve, muscle, M ϕ , megakaryocyte
			Epo, G-CSF	Mono specific, Erythroid, Neutrophil, T cells
		IFNR	IL-10, IFN- α/β	Immune modulation-cytokine synthesis
Long chain β -sheet	Cystine knot	S/T kinases	TGF- β s	Differentiation, growth inhibition, tissue repair
		Ig-TK	PDGF, VEGF	Epithelial and endothelial cell growth
		Cys-TK	NGF, BDNF	Survival, growth and differentiation of nerve cells

	β -jellyroll	TNFR	TNF- α/β , CD40L, FASL	Immune regulation, cell death
	β -trefoil	IL-1R (Ig-like)	IL-1 α/β , IL-18	Immune regulation, acute phase
Short chain α/β	S-S rich β -meander	Cys-TK	EGF, TGF α	Proliferation of epithelial cells, wound healing
	S-S rich α/β		Insulin, IGF-I/II	Metabolic responses mesenchymal
	Chemokines α/β	G-protein coupled	α -chemokines, IL-8, IP-10 β -chemokines, MCP-1/2/3, RANTES	Chemotaxis and Innate immune responses
Mosaic	Ig-EGF-TM-Cyt (cell surface)	Cys-TK	GGF	Growth and differentiation of nerve cells
	4-kringle (α) + serine protease (β)	Met-TK	HGF	Growth of hepatocytes, epithelial and endothelial cells
	4-helical (α) + hemopoietin (β)	Ig-TK	IL-12	Immune modulation T and NK cells

Legend: BDNF- brain-derived neurotrophic factor; CD40L- CD40 ligand; CNTF- ciliary neurotrophic factor; Cys-TK- cysteine rich tyrosine kinase; EGF- epidermal growth factor; Epo- erythropoietin; FASL- FAS ligand; G-CSF- granulocyte colony stimulating factor; GGF- Glial growth factor; GM-CSF- granulocyte macrophage colony stimulating factor; HGF- hepatocyte growth factor; IG-EGF-TM-CYT- immunoglobulin-like, epidermal growth factor, transmembrane and cytoplasmic domains; IGF- insulin-like growth factor; Ig-TK- immunoglobulin-like domain/tyrosine kinase domain; IFN- interferon; IFNR- interferon receptor; IL- interleukin; IL-1R- interleukin-1 receptor; MCP- macrophage chemotactic peptide; M-CSF- macrophage colony stimulating factor; Met-TK- methionine rich tyrosine kinase; M ϕ - macrophage; NGF- nerve growth factor; PDGF- platelet derived growth factor; SCF- stem cell factor; S/T- serine/threonine; TGF- transforming growth factor; TNF- tumour necrosis factor.

In view of the small degree of sequence homology between different cytokines, attempts to sub-classify these molecules have to rely on similarities on the induction mechanisms, overall three-dimensional structure, receptor subunits utilised and biological activities they stimulate (Nicola, 1994). A system for the classification of cytokines has been

proposed based on initial studies of protein structure (Bazan, 1990; Parry *et al*, 1991; Young, 1992). This organisation led to the establishment of four main categories of cytokines that were then subdivided on the basis of receptor utilisation. Groups of cytokines organised in this manner display common biological activities, as shown in table 1.2 (reviewed in Nicola, 1994).

The grouping of cytokines on the basis of their structure and receptor usage has revealed an evolutionary relationship amongst cytokines that would be impossible to disclose from biological activity and amino acid sequence data alone. It should be noted that the nomenclature of cytokines owes little to systematic relationships between the molecules. It is rather a consequence of the different approaches to naming new molecules, in areas of research as far afield as immunology and developmental biology, which are based either on the cell of origin or initial defining bioassay (Callard and Gearing, 1994).

1.3 Cytokine receptors

Cytokines bind high affinity receptors in order to exert their biological effects on target cells. The fact that individual cytokines bind to singular receptors has a bearing on the specificity of cytokine activity, but, on the other hand, highlights the multiplicity of receptor structures that exist to support such specificity. Nevertheless, purification of receptors and the cloning of genes encoding cytokine receptors has facilitated their grouping into families on the basis of shared structural features (table 1.3).

The most representative group of cytokine receptors is the class I cytokine receptor family also known as “hemopoietin receptors”. Members of this family are often multimers of receptor subunits, mostly heterodimers, but also homodimers (e.g G-CSFR and EpoR) and heterotrimers (e.g. IL-2R and IL-15R). Some members of this family form subfamilies by sharing the receptor chain responsible for the transduction of signal (β , γ or gp130 chain), a contributing factor in cytokine pleiotropy. The remaining chain(s) in each receptor are unique and act as specific binding components (Taga and Kishimoto, 1995; Heim, 1996).

Table 1.3 Classification of cytokine receptors (modified from Vilcek, 1998)

Receptor Family	Shared features	Ligands
Class I cytokine receptors	Conserved cysteines and WSXWS motif in the extracellular domain. Box1 and box2 in the intracellular domain	IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12, IL-13, IL-14, IL-15 GM-CSF, G-CSF, Epo, etc
Class II cytokine receptors	Conserved cysteines in the extracellular domain	IFN- α/β , IFN- γ , IL-10
TNF receptor family	Partial homology in the extracellular domain. Intracellular “death domains”	TNF- α/β , NGF, FASL, CD40L
IL-1 receptor family	Extracellular Ig-like domain	IL-1 α , IL-1 β , IL-1Ra, IL-18
TGF- β receptor family	Extracellular cysteine rich domains. Intracellular serine-threonine kinase domains	TGF- β
Chemokine receptor family	Seven transmembrane domains	IL-8, α and β chemokines

IL-1Ra - IL-1 receptor antagonist.

Members of the interferon receptor family are also termed class II cytokine receptors due to structural similarities to class I receptors. The major common feature to these two groups of receptors is the presence of 200 amino acid long domains containing conserved cysteine and proline residues and a series of hydrophobic and hydrophilic sequences also in conserved positions (Bazan, 1990).

The members of the TNF receptor family are single chain receptors that oligomerise by binding to their trimeric ligands. The main feature shared by some members of this group of receptors (e.g. TNF-R1, FAS) is the presence of cytoplasmic apoptosis domains (“death domains”) consisting of 90 amino acid sequences involved in protein-protein interaction with molecules (e.g. TNF-R associated factors - TRAFs) implicated in downstream signalling with activation of proteins such as caspases responsible for the deleterious effects of TNF- α and FAS ligand (reviewed in Wallach, 1997).

Members of the IL-1 receptor family are heterodimers of a ligand binding class I receptor subunit (IL-1R α chain), with the IL-1 receptor accessory protein (IL-1RAcP), representing the β chain essential for signalling (Greenfeder *et al*, 1995) although structural details are still to be fully understood.

The most distinctive feature that members of TGF- β R family share is the presence of a cytoplasmic serine-threonine kinase domain that makes these receptors unique. Other features such as the location of cysteine rich domains are the basis of the subdivision into type I (signalling component) and type II (ligand binding component) receptors (reviewed in Attisano and Wrana, 1996).

Members of the chemokine receptor family are also named G protein coupled receptors, mediating their effects through the interaction with guanine nucleotide-binding proteins (G proteins). The main structural feature of this group of receptors is the presence of seven hydrophobic motifs that produce transmembrane α helices (reviewed in Fraser *et al*, 1994).

1.4 Signal transduction

Following specific binding to receptors on target cells, cytokines promote transcriptional activation of responsive genes through the Jak-STAT pathway of intracellular signalling (reviewed in Gallin, 1995; Schindler and Darnell, 1995; Heim, 1996; Bach *et al*, 1997; Liu *et al*, 1998; Park and Schindler, 1998; Watanabe and Arai, 1998). The Jak-STAT pathway was first established in studies aimed at dissecting the mechanism of action of IFN- α and IFN- γ (Darnell *et al*, 1994). This system, now known to be involved in mediating cellular responses to most cytokines is composed of two distinct protein classes:

- the Janus kinases (Jak) are a family of soluble tyrosine kinases. Four members of this family have been identified to date (Tyk2, Jak1, Jak2 and Jak3) with molecular weights ranging between 125 and 135 kDa. These kinases share a sequence identity of 35 to 45% and an overall structural pattern (figure 1.1), with seven Jak homology (JH)

conserved domains, including two tandem tyrosine-kinase domains (JH1 and JH2). No function has been attributed to the remaining domains (JH3-JH7). A receptor binding region has been assigned to the N-terminal end of the molecule. JAKs have an ubiquitous distribution through most tissues. Only Jak3 expression appears to be restricted to cells of myeloid and lymphoid lineage. Although inactive in resting cells, Jaks are associated with cytokine receptor chains. Binding of ligand to the receptor leads to rapid phosphorylation of a tyrosine within the Jak kinase domain. Jaks have been found to associate and activate STATs.

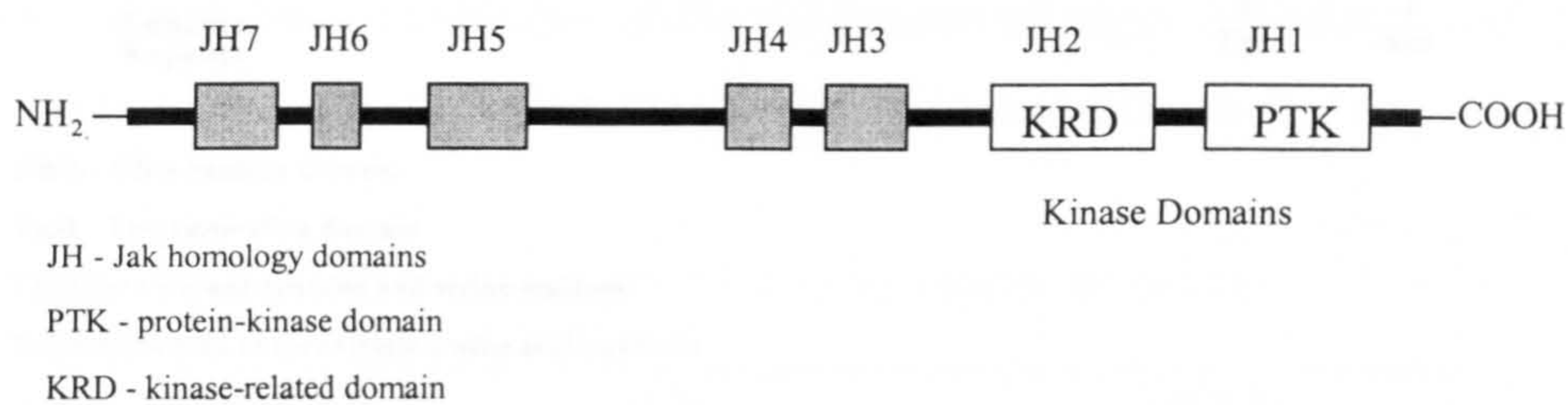


Figure 1.1 Structure of Janus family kinases (Heim, 1996; Bach *et al*, 1997).

- the signal transducers and activators of transcription (STATs) are a versatile group of proteins able to interact with receptor-kinase, generate homo or heterodimers, translocate to the nucleus and bind DNA in association or not with other nuclear proteins. STAT proteins are ubiquitous, with the exception of STAT4 expressed mainly in the thymus and testes. The number of STATs isolated to date is seven (STAT1-STAT6) with two isoforms of STAT5 (STAT5a, STAT5b). STAT proteins are 750 to 850 residues long and have an overall amino acid identity of 28 to 54%, with many conserved domains, as expected by the amount of functional requirements that they share. Common features (figure 1.2), identified by sequence analysis and deletion studies, include a single tyrosine at around position 700. Residues 600 to 700 show the strongest homology between STATs and correspond to a SH2 domain that binds phosphotyrosine and appears to determine which receptor will bind a given STAT protein (the STAT substrate is then phosphorylated regardless of which Jak kinases are associated with the receptor). Specific reciprocal SH2-phosphotyrosine interaction of two STATs leads to dimerisation that

precedes translocation to the nucleus. The 500 to 600 residue portion of the molecule is homologous to SH3 domains of other proteins but its function in the STATs has yet to be disclosed. Within residues 400 to 500 lies the DNA binding domain, responsible for gene transcription. Also, carboxy-terminal deletion studies have revealed a transactivation domain downstream the SH2 region. The only region with functional relevance in the N-terminus of STATs is a leucine repeat thought to facilitate interactions between family members.

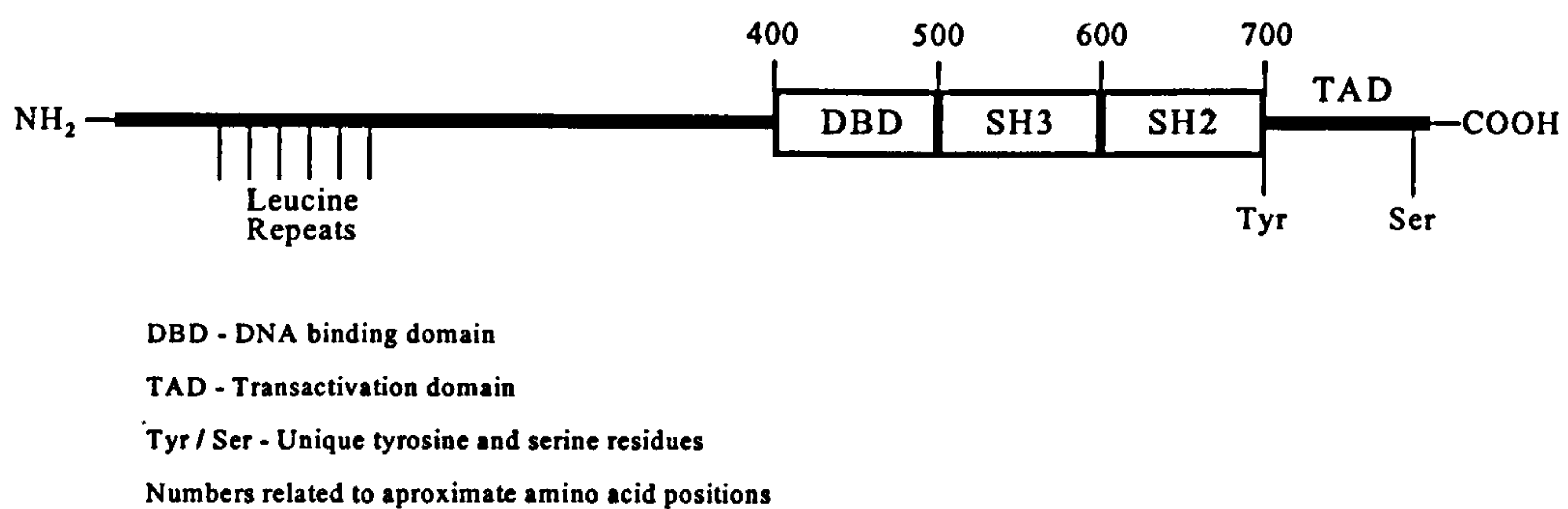


Figure 1.2 Structure of STAT proteins (Heim, 1996, Bach *et al*, 1997).

Dimeric STATs activate gene transcription by binding to responsive elements in the promoters of genes to be transcribed, following direct or indirect association with the RNA polymerase II complex. DNA motifs that bind STATs can be classified in two groups. The first group is represented by the interferon-stimulated responsive element (ISRE) and share the consensus sequence AGTTTCNNTTTCNC/T. The second group has the IFN- γ activation site (GAS) as a prototype and share the palindromic core sequence TTNNNNNAA, where variations of the inner residues were shown to affect differential binding to STATs.

In view of the restricted numbers of both Jaks and STATs and of the growing number of receptors that seem to signal via this system, it is clear that the specificity of cytokine signal transduction is not due to dedicated signalling molecules. Rather, it is particular combinations of Jaks and STATs which expand signalling variability. On table 1.4 is shown the Jak and STAT usage by cytokine receptors.

Factors that increase diversity and therefore determine specificity of the signal include the selective activation of STATs by ligands, the STAT versatility and the variability at the gene level. By binding only to specific sets of STATs the receptors transmit into the cell the specificity of the ligand-receptor interaction. By being able to heterodimerize selectively and interact with non-STAT proteins, STATs increase the potential of specific ligand responses. Due to the variability of ISRE and GAS responsive elements it is plausible that different STATs (or STAT combinations) may be able to discern between these elements and specifically activate certain genes. The Jak-STAT pathway has therefore the potential for specific cytokine signal transduction from the cell membrane to the genes. For a detailed description of the mechanism of signal transduction of IFN- γ , which constitutes a model of cytokine signalling, can be found below (1.7.4).

Table 1.4 Activation of Jaks and STATs by various cytokines
(modified from Liu *et al*, 1998)

Cytokine	Jaks	STATs
IFN- α	Tyk2 / Jak1	STAT 1/2/3/5
IFN- γ	Jak1 / Jak2	STAT 1
IL-2	Jak1 / Jak3	STAT 5
IL-3	Jak2	STAT 5
IL-4	Jak1 / Jak3	STAT 6
IL-5	Jak2	STAT 5
IL-6	Tyk2 / Jak1 / Jak2	STAT 1/3
IL-7	Jak1 / Jak3	STAT 5
IL-9	Jak1 / Jak3	STAT 5
IL-10	Tyk2	STAT 3/5
IL-11	Jak1	STAT 1/3
IL-12	Tyk2 / Jak2	STAT 1/3/4
IL-13	Jak1	STAT 6
IL-15	Jak1	STAT 5
GM-CSF	Jak2	STAT 5
G-CSF	Jak1/ Jak2	STAT 1/5
Epo	Jak2	STAT 5
EGF	Jak1	STAT 1/3

1.5 The Th1 / Th2 paradigm

The specific immune response to a microbial pathogen is an active process that depends on factors derived from both the microorganism and the host. An appropriate immune

response leads to the eventual clearance of infection and generation of an effective memory, whereas an inappropriate response results in a lack of protection and in some cases even exacerbates the pathological process (Kemp *et al*, 1996). Many bacterial, protozoal and viral infections trigger cell-mediated immune responses, while other pathogens (such as helminths) or their products induce primarily a humoral response (Del Prete and Romagnani, 1994).

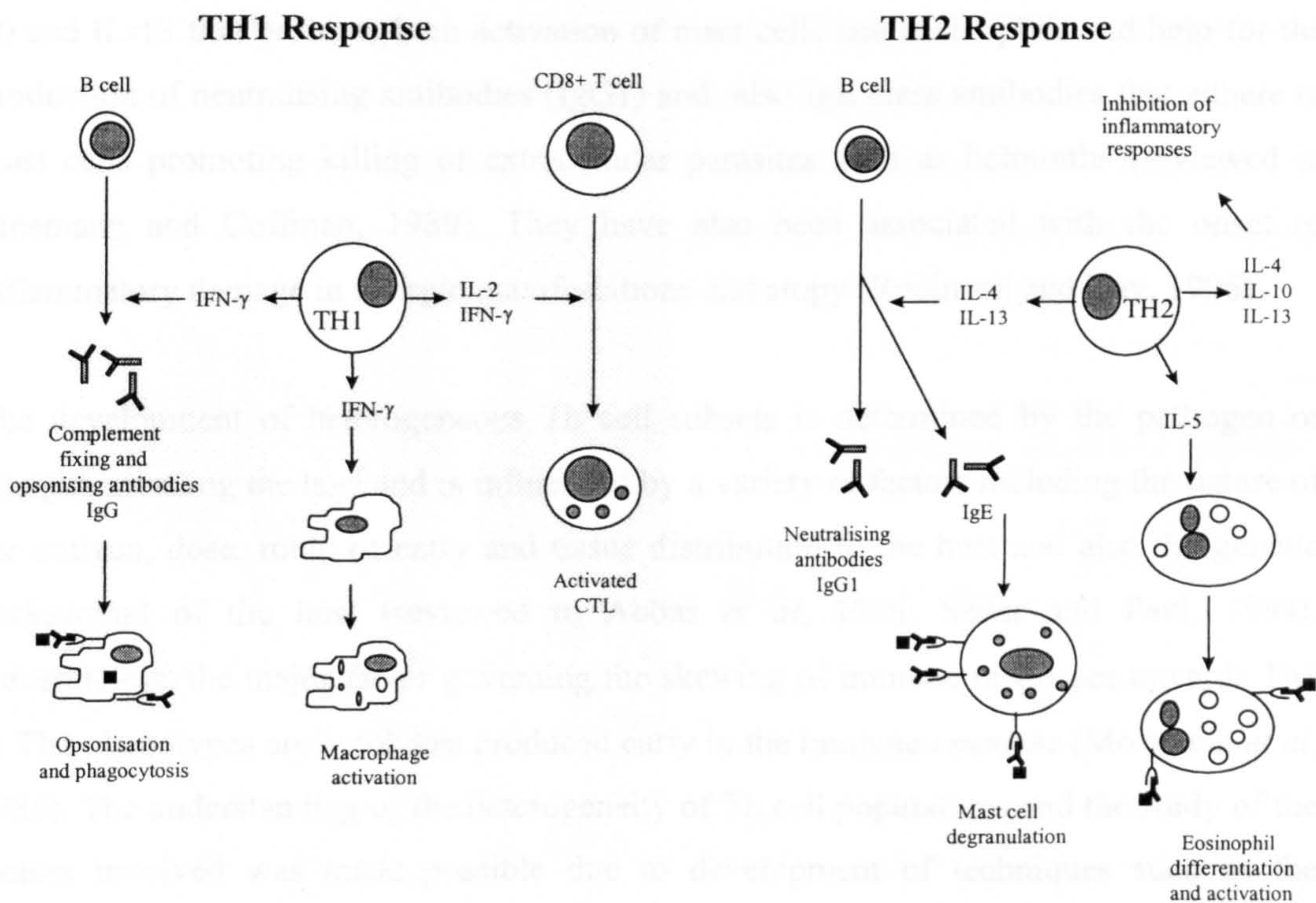


Figure 1.3 Functions of CD4+ Th1 and Th2 subsets (modified from Abbas *et al*, 1996).

Investigation of the murine immune system revealed distinct subsets of T helper cells with roles in cell mediated and humoral responses to antigen, respectively Th1 and Th2 cells. The recognition that CD4+ T cells (T helper cells), by exhibiting discrete patterns of cytokine expression, can activate different immunological effector mechanisms (figure 1.3), offered an explanation for the differential immune responses to antigen observed in infectious processes (Mosmann *et al*, 1986).

Two major T helper (Th) subsets have been recognised. Th type 1 (Th1) cells produce IL-2, IFN- γ and lymphotoxin (TNF- β), promoting macrophage activation leading to delayed type hypersensitivity and also complement fixing and opsonizing antibodies required to clear infection by intracellular organisms (reviewed in Mosmann and Coffman, 1989). Th1 cells have been implicated, when inappropriately activated, in the immunopathology of organ specific autoimmune diseases (e.g. insulinitis in insulin dependent diabetes mellitus) (Fowell and Mason, 1993). The type 2 (Th2) cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13 that promote both activation of mast cells and eosinophils and help for the production of neutralising antibodies (IgG1) and also IgE class antibodies that adhere to mast cells promoting killing of extracellular parasites such as helminths (reviewed in Mosmann and Coffman, 1989). They have also been associated with the onset of inflammatory damage in allergic manifestations and atopy (Robinson and Kay, 1996).

The development of heterogeneous Th cell subsets is determined by the pathogen or allergen invading the host and is influenced by a variety of factors including the nature of the antigen, dose, route of entry and tissue distribution in the host and also the genetic background of the host (reviewed in Abbas *et al*, 1996; Seder and Paul, 1994). Nevertheless, the major factor governing the skewing of immune responses towards Th1 or Th2 phenotypes are cytokines produced early in the immune response (Mosmann *et al*, 1986). The understanding of the heterogeneity of Th cell populations and the study of the factors involved was made possible due to development of techniques such as the propagation and activation *in vitro* of T cell clones, transgenic models and assays for cytokine detection and quantitation (e.g. capture ELISA, Elispot and intracellular staining of cytokines).

When naive Th cells encounter activated antigen presenting cells (APCs), cross linking of the T cell receptor (TCR) with antigen presented in the context of major histocompatibility complex class II (MHC II), occurs. This results in T cell activation denoted by the production of IL-2 (Abbas *et al*, 1996). Also the expression by APCs of costimulatory molecules, such as B7 (B7-1 and B7-2) that binds to CD-28 expressed by T cells, is necessary for full T cell activation (reviewed in Constant and Bottomly, 1997).

When antigen presentation occurs in the presence of IL-12, the Th cells develop into the Th1 phenotype (figure 1.4) of IFN- γ producing cells. IL-12 is produced by activated macrophages and dendritic cells in response to potent microbial adjuvants such as endotoxin and intracellular bacteria (e.g. *Listeria*, mycobacteria), protozoa (e.g. *Toxoplasma*) and viral components (Trinchieri, 1995). Although IL-12 is the most important factor in driving Th1 responses, IL-1 α was also identified as a cofactor in BALB/c mice. Of importance seems to be also the newly defined cytokine IL-18 (formerly known as IGIF for IFN- γ inducing factor) that synergises with IL-12 in the induction of IFN- γ , suggesting that both IL-12 and IL-18 are needed for optimum Th1 development (reviewed in Abbas *et al*, 1996; O’Garra and Murphy, 1996; O’Garra, 1998).

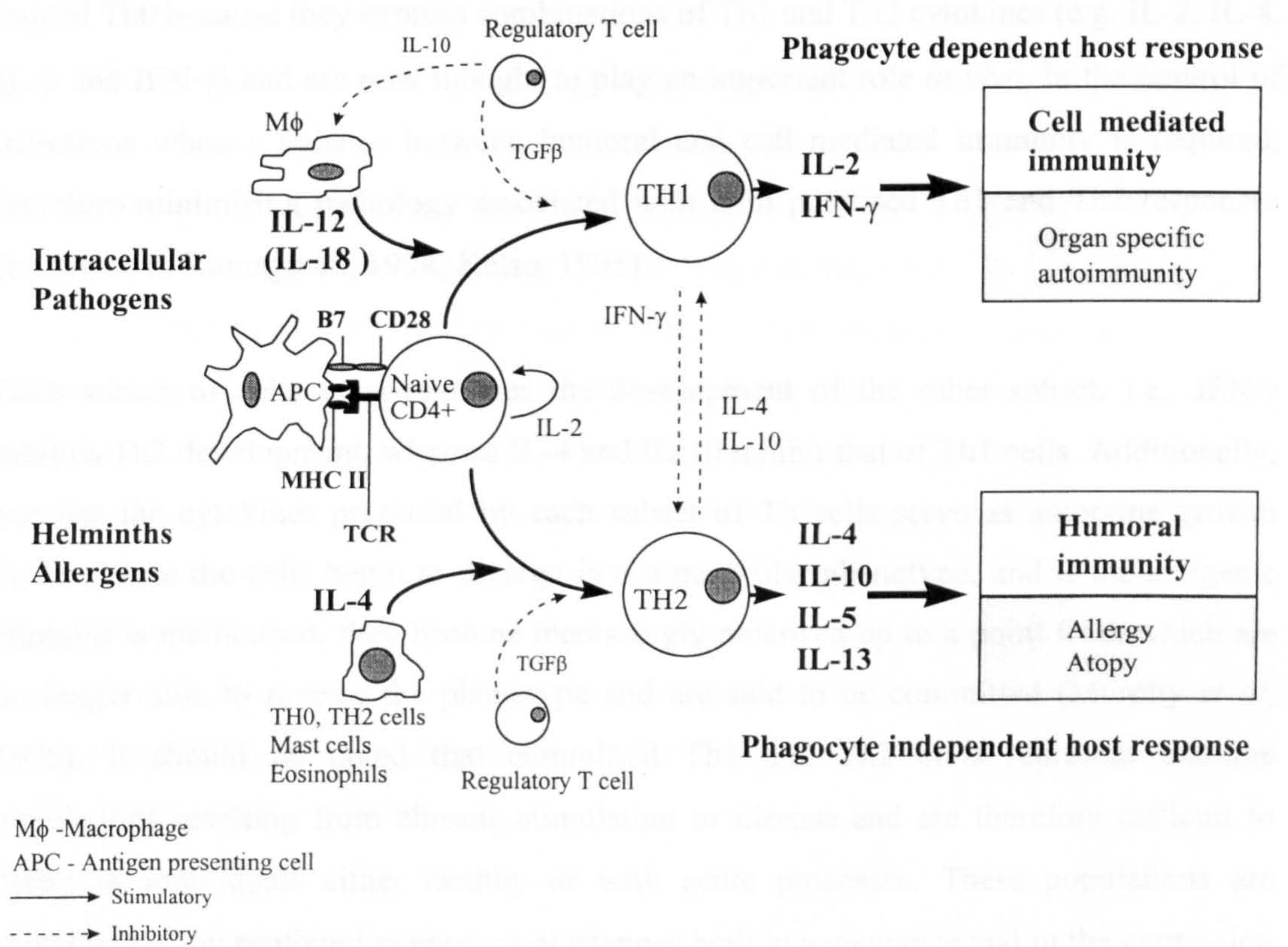


Figure 1.4 Regulation of Th cell responses by cytokines (modified from O’Garra, 1998).

Conversely, when naive Th cells encounter antigen in the presence of IL-4 they develop into a Th2 phenotype (figure 1.4). Therefore, IL-4 is both required for Th2 development and expressed by Th2 cells. The main producers of IL-4 are the CD4⁺ T cells themselves (possibly memory and even naïve cells) although there is evidence other cells including mast cells, basophils, eosinophils and certain subsets of NK cells (Paul *et al*, 1993) produce IL-4, therefore playing a role in directing Th2 development (reviewed in Abbas *et al*, 1996; O'Garra, 1998). The activity of IL-4 is dominant over that of Th1 inducing cytokines, resulting that when the level of IL-4 reaches a certain threshold Th2 will differentiate despite the presence or not of IL-12 and associated cytokines.

This Th1/Th2 dichotomy is far from absolute. When analysing cells from normal individuals rather than clones or cells from transgenic models, a large percentage of individual cells exhibit heterogeneous patterns of cytokine expression. These cells were named Th0 because they express combinations of Th1 and Th2 cytokines (e.g. IL-2, IL-4, IL-5 and IFN- γ) and are now thought to play an important role *in vivo*, in the control of infections when a balance between humoral and cell mediated immunity is required, therefore minimising pathology associated with both polarised Th1 and Th2 responses (reviewed in Romagnani, 1994; Kelso, 1995).

Each subset of cells cross-regulates the development of the other subset, i.e., IFN- γ inhibits Th2 development, whereas IL-4 and IL-10 inhibit that of Th1 cells. Additionally, because the cytokines produced by each subset of Th cells serve as autocrine growth factors, once the cells begin to diverge into a particular phenotype, and if the antigenic stimulus is maintained, they become increasingly polarised up to a point from which are no longer able to reverse the phenotype and are said to be committed (Murphy *et al*, 1996). It should be noted that committed Th1 and Th2 cells represent extreme populations resulting from chronic stimulation or disease and are therefore difficult to detect in individuals either healthy or with acute processes. These populations are characterised by profound phenotypical changes both in appearance and in the expression of specific markers. For example Th2 cells downregulate the expression of IL-12R and

IL-18R, whereas in Th1 although IL-4 receptor expression is maintained tyrosine phosphorylation by this receptor is impaired (Anne O'Garra personal communication).

Also, *in vivo*, the nature and dose of the antigen appears to have a bearing on the outcome of Th development. Antigen in low doses tends to be processed by professional APCs (e.g. dendritic cells) that express IL-12 and therefore drive Th1 development. On the other hand antigen in high doses is also rescued by alternative APCs (e.g. B cells) that do not express IL-12, favouring Th2 polarisation (Abbas *et al*, 1996). An additional parameter of control is offered by the so called regulatory T cells (e.g. TR1) which by expressing TGF- β and IL-10 are able to quench both Th1 and Th2 development and associated pathologies (Weiner, 1997).

1.6 Cytokines as vaccine adjuvants

Safety concerns related with the use of whole inactivated or attenuated pathogens for vaccination have precipitated the development of subunit vaccines comprising representations of antigenic components of pathogens by synthetic peptides, recombinant proteins and, lately, genes delivered both in minimal virus vectors or plasmid DNA. Although much safer, such vaccines are generally less immunogenic than live attenuated vaccines and there is a continued search for substances that can boost immune responses and therefore be used as vaccine adjuvants (reviewed in Edelman, 1997). Many substances have been proposed as adjuvants, although, because of potential toxicity, only a limited number as been approved for clinical use (Gupta *et al*, 1993; Vogel and Powell, 1997). The fact that most adjuvants (e.g. LPS, Freund's adjuvant) mediate their activity through nonspecific induction of cytokines and the accumulated knowledge of cytokine biology and immune modulatory functions has prompted the use of cytokines as vaccine adjuvants (Dong *et al*, 1997).

Cytokines have two major advantages when compared to other adjuvants. Firstly, because cytokines are natural host products are less likely to promote undesirable side effects. Secondly, their specific immunomodulatory activities include enhancement of antigen

presentation, activation and growth of T or B cells and appropriate shifting of the immune response towards cell mediated (Th1) or humoral (Th2) responses (Lin *et al*, 1995; Dong *et al*, 1997). Among the cytokines more thoroughly tested as adjuvants are those that are effective when injected in a single dose near the time of antigen inoculation (e.g. IL-1, IL-2 and IFN- γ). The rationale for using these cytokines and examples of their use are extensively reviewed in Heath and Playfair, 1992; Schijns *et al*, 1994; Lin *et al*, 1995 and Heath, 1997 as summarised in table 1.5.

Table 1.5 Effects of cytokines as vaccine adjuvants (modified from Cohen *et al*, 1998).

Cytokine		Effect on the immune response	
Type	Name	Ab production	CTL activity
Proinflammatory	IL-1	↑↑	↑↑
	TNF- α	↑↑	↑↑
	TNF- β	↑↑	↑↑
	GM-CSF	↑↑	↑↑
Th1 inducing	IL-2	↑↑	↑↑
	IL-12	↓	↑↑
	IL-15	↑↑	↑↑
	IL-18	↑↑	↑↑
	IFN- γ	↓	↑↑
Th2 inducing	IL-4	↑↑	↓
	IL-5	↑↑	↔
	IL-10	↑↑	↔

Ab- antibody; ↑↑ -increased response; ↔ -no change in response; ↓-decreased response.

These studies highlight the importance of the awareness not only of the specific functions of individual cytokines but also of the type of immune response that is required for protection. Eliciting the wrong response might not only be futile in terms of vaccine efficiency but also be responsible for an increase of severity of the infectious process upon challenge with live pathogen.

In a veterinary context, the use of cytokines as adjuvants is dependent on the production of pure preparations of recombinant cytokines, a costly and time consuming procedure, due to the non-availability of commercial species-specific molecules. The advent of gene

therapy has however facilitated the delivery of cytokines in a cheap and easy way, which is reflected by the number of experiments where cytokines were used individually or in various combinations to boost immune responses to co-administered encoded antigen (reviewed in Cohen *et al*, 1998; Kim *et al*, 1998). In one of such studies (Chow *et al*, 1998) the potential for the use of cytokines to shift the nature of immune responses to Hepatitis B DNA vaccines in a murine model was evaluated. Mice were immunised with plasmid DNA encoding HBV major envelope proteins co-administered with various plasmids encoding cytokine genes. The results obtained are summarised in table 1.6.

Table 1.6 Cytokine adjuvancy of hepatitis B DNA vaccination (summarised from Chow, 1998).

Plasmid encoding HBV envelope protein plus:	T helper response (as measured by cytokine profile of splenocytes)	Antibody response (in terms of IgG isotype)	CTL response (as accessed by % of specific lysis)
Plasmid encoding IFN- γ or IL-12	Th1 enhanced Th2 inhibited	IgG2a increased IgG1 decreased	between 35 and 50%
Plasmid encoding IL-4	Th1 suppressed Th2 enhanced	IgG2a suppressed IgG1 increased	less than 20%
Plasmid encoding IL-2 or GM-CSF	Th1 enhanced Th2 not affected	IgG2a increased IgG1 increased	between 25 and 35%

It can be concluded that while IL-12 and IFN- γ favoured a cell mediated immune response, IL-4 favoured humoral immunity whereas IL-2 and GM-CSF had an intermediate effect. The delivery of cytokine genes in DNA vaccination formulations can therefore influence the differentiation of Th subsets and consequently the type of immune response.

1.7 Interferon gamma

Interferon gamma (IFN- γ) or immune-interferon belongs to a family of proteins related by their ability to protect cells from viral infection (Farrar and Schreiber, 1993). This activity requires *de novo* synthesis of RNAs and proteins by stimulated cells (Borden, 1992).

Interferons (IFNs) are classified in type I and type II based on primary structure similarities and receptor binding studies.

Type I IFNs include IFN- α and IFN- ω (formerly known as leucocyte IFN) and IFN- β (fibroblast IFN) that share extensive amino acid sequence homology and bind to a common cell surface receptor. IFN- α constitutes, in itself, a complex family of genes and pseudogenes, encoding over 22 distinct proteins (Farrar and Schreiber, 1993 ; Donnelly, 1994). Type I IFN genes do not contain introns and are clustered in a unique chromosome: human chromosome 9 and murine chromosome 4. These genes code for proteins with a 23 amino acid signal peptide and mature proteins between 164 and 166 residues in length. These IFNs share a potent antiviral activity and moderate antiproliferative and immunomodulatory activities (Donnelly, 1994). A further member of the Type I family is the trophoblast interferon (IFN- τ) of domestic ruminants. IFN- τ is expressed early in embryogenesis and may therefore play a role in the implantation process and early stages of the embryo development (Roberts *et al*, 1991)

IFN- γ (or immune interferon) is the sole Type II IFN. It binds to a distinct receptor and is completely unrelated at both genetic and protein levels to Type I IFNs. IFN- γ , although originally defined as an antiviral agent (Wheelock, 1965), is primarily an immunomodulatory peptide, playing a central role in the regulation of several aspects of an effective immune response and, consequently, in the resistance of mammalian hosts to pathogens.

1.7.1 Molecular Structure

IFN- γ is encoded by a single gene, mapped to chromosome 12 (12q24.1) in humans (Naylor *et al*, 1983) and chromosome 10 in the mouse (Naylor *et al*, 1984). Both human and murine genes are 6 Kbp in size and contain four exons and three introns.

Among the main features of the gene are the Goldberg-Hogness element (TATA box) with the sequence TATAAATA, which is located approximately 30bp upstream from the

transcription start and is thought to be important for determining the specificity of the initiation of transcription. The sequence (GTCACCATCT) located 90bp upstream from the transcription start, is similar to the consensus $GG^C/TCAATCT$ (CAAT box) relatively well conserved in the 5' end of many genes and involved in the modulation of transcription by RNA polymerase II (Gray and Goeddel, 1982). Other features include the transcription start, the polyadenylation signal sequence (AATAAA) and the polyadenylation site (Figure 1.5).

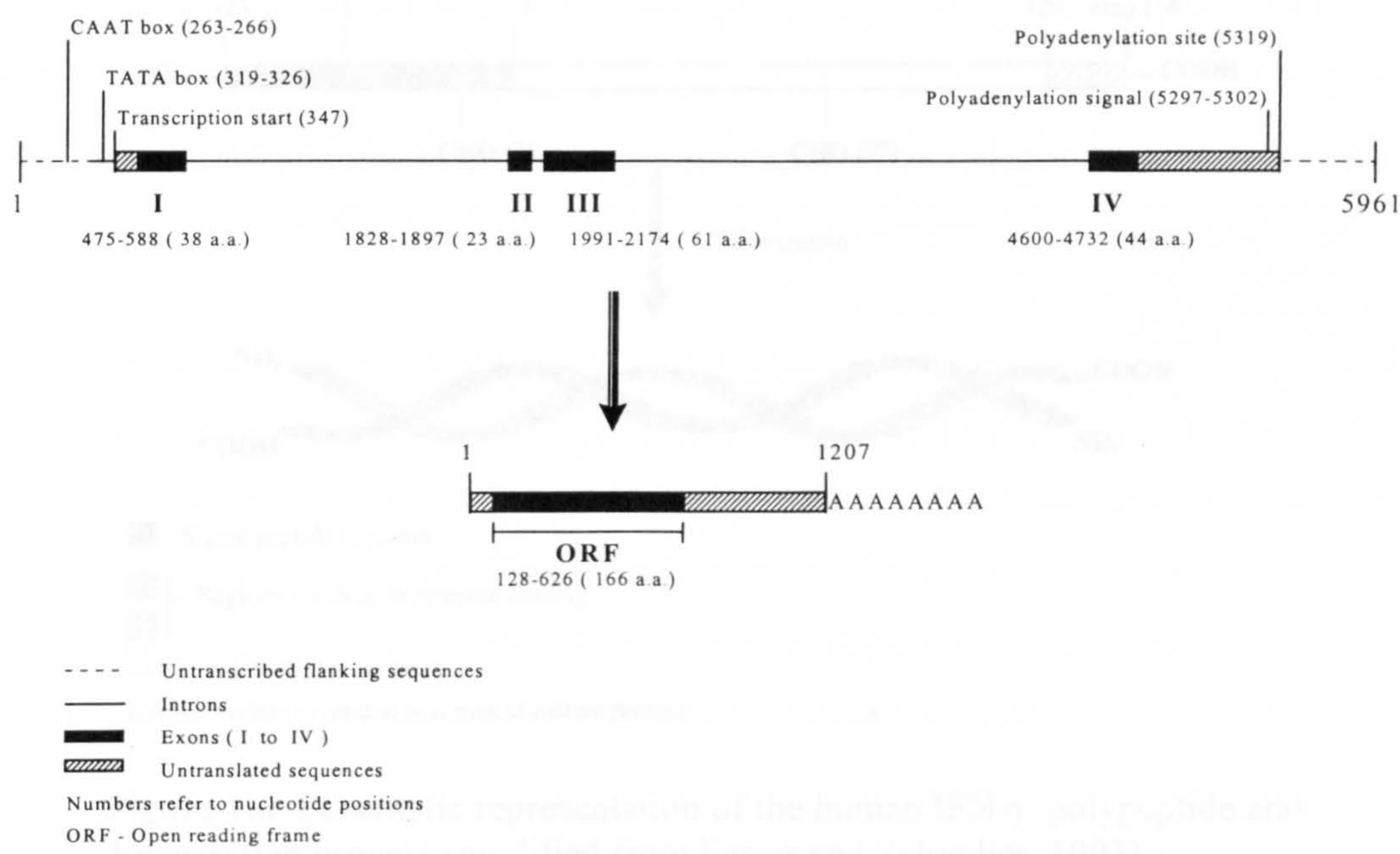


Figure 1.5 Representation of the human IFN- γ gene structure and generation of the corresponding mRNA molecule (Gray and Goeddel, 1982).

DNase I hypersensitivity and deletion analyses of the IFN- γ gene uncovered an array of sequences implicated in the regulation of transcription. These include a 600bp region (within the core promoter) upstream of the TATA box displaying a pattern of enhancer-silencer- enhancer elements. Another enhancer region was found within the first intron of the gene, functional in both T cells and fibroblasts but appearing to be inducible only in T cells. A further six elements capable of interacting with members of the NF κ B family of

DNA binding proteins were detected in introns 1, 2 and 3. (Hardy *et al*, 1987; Ciccarone *et al*, 1990; Young, 1995).

Activation of the gene leads to transcription, splicing and polyadenylation events, rendering a 1.2Kbp mRNA transcript containing an open reading frame encoding a 166 amino acid polypeptide. The human IFN- γ polypeptide chain (Figure 1.6) is extensively modified prior to secretion.

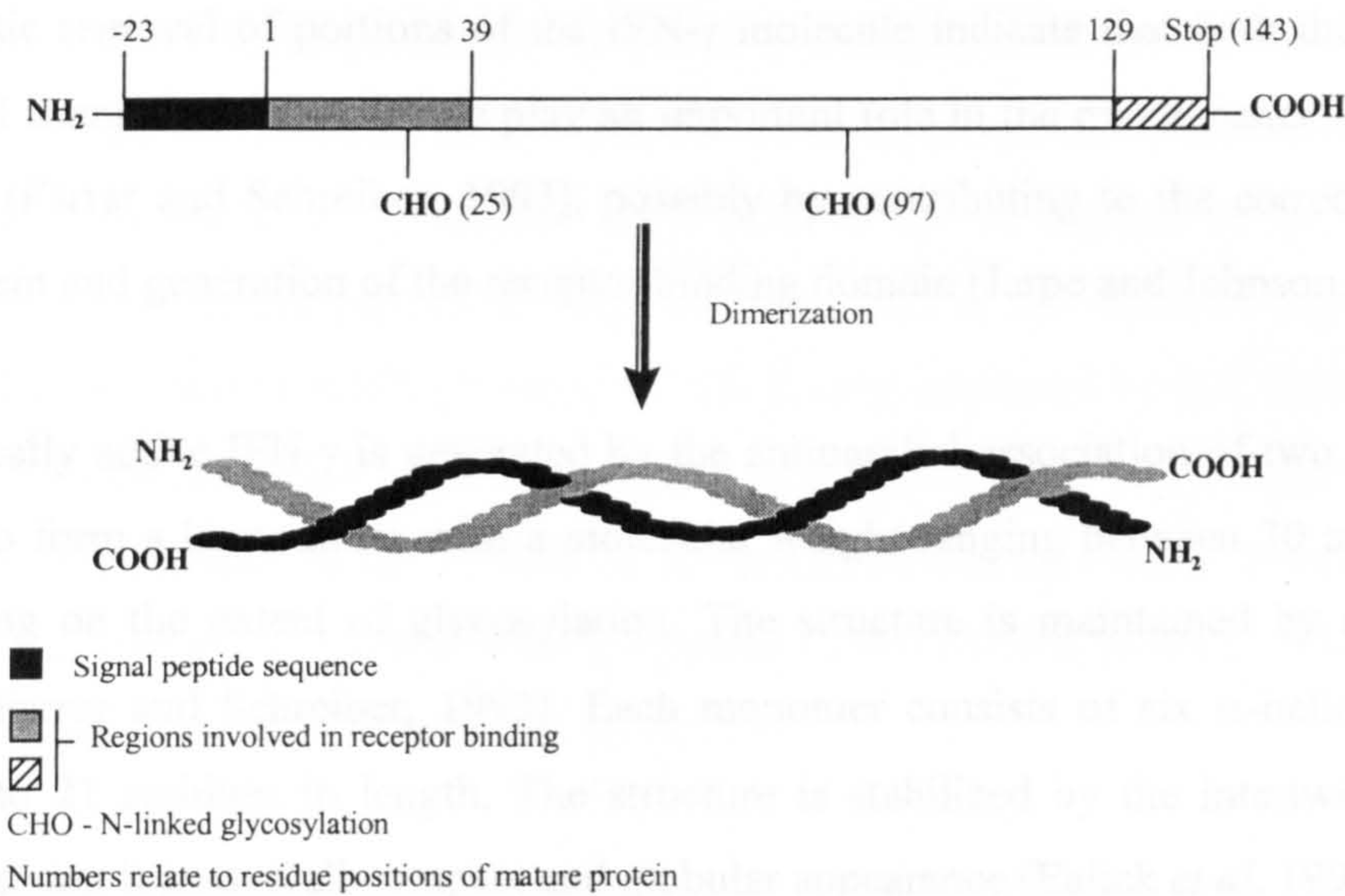


Figure 1.6 Schematic representation of the human IFN- γ polypeptide and dimerisation process (modified from Farrar and Schreiber, 1993).

Post-translational modifications include N-linked glycosylation, signal peptide cleavage, cytoclisation of the amino terminal glutamine into pyroglutamic acid and enzymatic degradation of the carboxyl terminus leading to variable length of the resulting peptide. Up to nine residues can be removed without significant loss of biological activity (Gray, 1994). Proteolytic cleavage of the hydrophobic leader sequence (amino terminal 23 residues) gives rise to a positively charged 143 residue mature polypeptide with an apparent molecular weight of 15 KDa under denaturing conditions (Gray *et al*, 1982; Rinderknecht *et al*, 1984). The human IFN- γ polypeptide contains two potential N-linked glycosylation sites at residues 25 and 97. Natural IFN- γ analysed under denaturing

conditions as an apparent molecular weight of 25 Kda when fully glycosylated. Nevertheless molecules of 20 Kda, corresponding to the glycosylation of only one site (generally position 25) have also been detected (Rinderknecht *et al*, 1984). Glycosylation is not required for the development of biological activity but appears to influence clearance of the molecule from circulation and therefore its half-life (Farrar and Schreiber, 1993).

Studies employing oligopeptide specific monoclonal antibodies, synthetic peptides and enzymatic removal of portions of the IFN- γ molecule indicate that both the amino and carboxyl termini of the molecule play an important role in the maintenance of biological activity (Farrar and Schreiber, 1993), possibly by contributing to the correct folding of the protein and generation of the receptor binding domain (Jarpe and Johnson, 1990).

Biologically active IFN- γ is generated by the antiparallel association of two polypeptide chains to form a homodimer with a molecular weight ranging between 30 and 50 KDa, depending on the extent of glycosylation. The structure is maintained by noncovalent forces (Farrar and Schreiber, 1993). Each monomer consists of six α -helices, ranging from 9 to 21 residues in length. The structure is stabilized by the intertwining of the helices giving it an overall compact and globular appearance (Ealick *et al*, 1991).

1.7.2 Production of IFN- γ

The main cellular producers of IFN- γ are activated natural killer (NK) cells (Perussia, 1991) and, to a greater extent, activated (CD30 expressing) T cells (Alzona *et al*, 1994). Moreover, IFN- γ production is the basis of the concept of type 1/ type 2 dichotomy in cytokine production, originally described by Mosmann *et al* (1986) for mice T helper cells and followed by similar observations in humans (reviewed in Mosmann and Coffman, 1989; Mosmann and Sad, 1996). These observations show that T helper cells exhibit discrete patterns of cytokine production, with IFN- γ secretion associated with Th1 cells (discussed in 1.5). More recently, this pattern of cytokine release was observed in other T cell subsets including CD8⁺ (TC1 cells) and TCR γ/δ T cells (T $\gamma\delta$ 1 cells)

(reviewed in Carter and Dutton, 1996). Uncommitted Th cells (Th0) were also reported to produce significant amounts of IFN- γ under *in vitro* stimulation conditions (O'Garra, personal communication). As of late, a significant amount of evidence, gathered from both *in vivo* and *ex vivo* studies, points towards expression of IFN- γ by other cellular types, namely anti-CD40 activated B cells (Yoshimoto *et al*, 1997) and bone marrow-derived macrophages (Munder *et al*, 1998), upon combined stimulation with IL-12 and IL-18.

The mechanism of IFN- γ production is linked to the differential activation pathways that IFN- γ producing cells exhibit. In the case of NK cells, activation occurs through binding of the NK cell low affinity receptor (Fc γ RIIA) to the Fc regions of IgG1 and IgG3 molecules bound to the surface of target cells, as part of the antibody-dependent cell-mediated cytotoxicity (ADCC) method of cell killing employed by NK cells (Abbas¹ *et al*, 1997). T cell activation requires specific interaction of the T cell receptor (TCR α/β) with antigen presented in the context of the major histocompatibility complex (MHC). CD8⁺ CTLs interact with MHC class I, whereas CD4⁺ Th cells interact with MHC class II (Farrar and Schreiber, 1993; Abbas² *et al*, 1997).

The expression of costimulatory molecules (e.g. B7, ICAM-1) by APCs is of paramount importance for the correct activation of T cells. Binding of B7 to CD28 (expressed by resting T cells) enhances T cell responses to antigen. In contrast, binding of B7 to CTLA-4 (expressed in a percentage of activated T cells) arrests T cell activation and promotes apoptosis and is therefore thought to play a role in the termination of T cell responses. In the absence of costimulatory signals the T cell remains anergic, contributing for the maintenance of tolerance to self antigens (reviewed in Lenschow *et al*, 1996).

A second set of mechanisms of activation and IFN- γ secretion common to both NK and T cells, is mediated by APCs by one or more of the following methods:

- the APC (target cell) interacts through the adhesion molecules LFA-3 and ICAM-1 with CD2 and LAF-1, respectively, on the surface of the lymphocyte as part of target cell recognition (Freedman *et al*, 1991; Wingren *et al*, 1993);

- APCs process bacteria (e.g. *S. aureus*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Chlamydia*); bacterial products (e.g. LPS from *Salmonella typhi*); protozoa (e.g. *Leishmania major*, *Trypanosoma cruzi*); viruses (e.g. *Influenza*) and fungi (e.g. *Candida*) and secrete cytokines. These cytokines in conjunction with soluble pathogen products (in the case of NK cells) or peptides presented in the context of the MHC (for T cells) will serve as a signal for cell activation and secretion of IFN- γ (Morris *et al*, 1982; Heremans *et al*, 1994; Szein *et al*, 1994; D'Orazio *et al*, 1995; Flesh *et al*, 1995). Additionally, products of activated T cells and macrophages (e.g. hydrogen peroxide and leukotrienes - LTC₄, LTD₄, LTE₄) enhance IFN- γ secretion (Farrar and Schreiber, 1993).

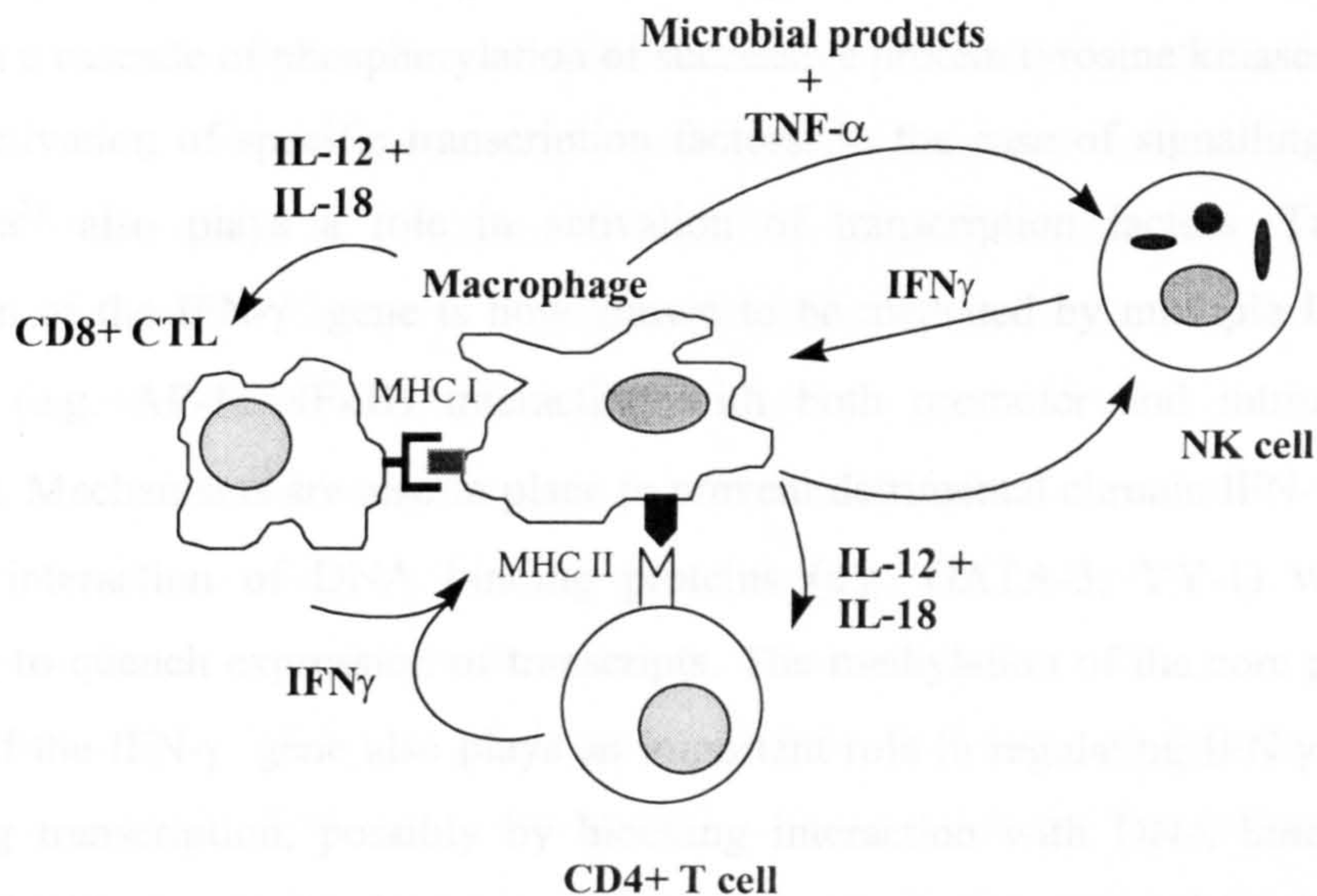


Figure 1.7 Cellular sources and main stimuli for the production of IFN- γ , showing the reciprocal induction of IFN- γ and IL-12/IL-18 (modified from Vilcek and Le, 1993; Farrar and Schreiber, 1993).

The cytokines IL-1 α , TNF- α , IL-2, IL-6, IL-12 and IL-18 are required for optimal IFN- γ production. IL-2 with its autocrine and paracrine T cell growth factor activity and IL-6 acting as costimulator of T cells, induce increased production of IFN- γ by the effect they have in T cell proliferation and degree of activation (Abbas^{3,4} *et al*, 1997). In contrast, IL-12, by directly skewing CD4+ cells towards a Th1 phenotype and IL-1 α , TNF- α and

especially IL-18 by their synergism with IL-12, are responsible for a dramatic increase in the local secretion of IFN- γ (reviewed in Romagnani, 1994; Fearon and Locksley, 1996; Mosmann and Sad, 1996; Okamura *et al*, 1998; Shibuya *et al*, 1998). IFN- γ production can also be induced experimentally by direct stimulation of the T cell receptor complex with anti-CD3 antibodies, by mitogens such as Concanavalin A (ConA), Lipopolysaccharide (LPS) and Phytohemagglutinin (PHA) or by a combination of phorbol myristate acetate (PMA) and ionomycin (Farrar and Schreiber, 1993).

Lymphocyte activation and regulation of IFN- γ production is a tightly controlled and complex process involving soluble extracellular signals (e.g. cytokines) and cell contact (e.g. TCR) (reviewed in Young, 1996; Young and Ghosh, 1997). Cellular activation results in a cascade of phosphorylation of successive protein tyrosine kinases culminating in the activation of specific transcription factors. In the case of signalling through the TCR, Ca^{2+} also plays a role in activation of transcription factors. Transcriptional activation of the IFN- γ gene is now known to be mediated by multiple DNA binding proteins (e.g. AP-1, NF κ B) interacting with both promoter and intronic enhancer elements. Mechanisms are also in place to prevent detrimental chronic IFN- γ production through interaction of DNA binding proteins (e.g. GATA-3, YY-1) with supresor elements to quench expression of transcripts. The methylation of the core promoter and introns of the IFN- γ gene also plays an important role in regulating IFN- γ production, inhibiting transcription, possibly by blocking interaction with DNA binding proteins (reviewed in Young, 1995). Post-transcriptional events are also critical for the expression of this cytokine as illustrated by the stabilisation of IFN- γ mRNA mediated by activation of protein kinase C and increased intracellular levels of cAMP induced by IL-2 (Kaldy *et al*, 1995).

Studies on the kinetics of IFN- γ expression and secretion show that following *in vitro* stimulation of T cells, IFN- γ mRNA starts accumulating after 6 to 8 hours, peaking between 12 to 24 hours post stimulation. The secreted protein can be readily detected in the cellular medium 8 to 12 hours post stimulation and peaking after 18 to 24 hours.

Moreover, because IFN- γ is not significantly consumed by the cells in culture, it remains detectable long after the T cell activation status declines (Schreiber *et al*, 1983).

1.7.3 Interferon Gamma Receptor

In order to exert its pleiotropic biological activities, IFN- γ must bind to specific receptors expressed on the surface of susceptible cells. This interaction is one of high affinity (K_a of 10^9 to 10^{10} M $^{-1}$) as determined by radioligand binding experiments (Mao *et al*, 1989). The cloning of human and murine IFN- γ receptor (IFN- γ R) cDNA (Aguet *et al*, 1988; Gray *et al*, 1989) enabled the study of its structure and function.

The IFN- γ R is composed of two subunits formed by distinct polypeptide chains, α -chain and β -chain. The α -chain is encoded by a 30 Kbp gene located on the chromosome 6 in humans (Pfizenmaier *et al*, 1988) and by a 22 Kbp gene of chromosome 10 of the mouse (Mariano *et al*, 1987). The gene contains a noninducible promoter (with no TATA box) and seven exons. Transcription of the human gene gives rise to a 2.3 Kb mRNA (Farrar and Schreiber, 1993). The β -chain gene is located on human chromosome 21 and murine chromosome 16 and contains seven exons and several potential binding sites for transcription factors in the promoter region. Transcription of the gene generates an mRNA transcript of 1.8 Kb in the human and 2 Kb in the mouse (Hibino *et al*, 1991; Cook *et al*, 1994). Both polypeptide chains are synthesized in the ER and post-translationally modified in the Golgi by extensive N-glycosylation in 5 or 6 sites, respectively in the α and β -chain (Mao *et al*, 1989).

Nearly all somatic cells, with the exclusion of erythrocytes, are able to bind IFN- γ . However while most cells express vast amounts of the receptor α -chain (ranging from 200 to 25000 sites per cell) (Farrar and Schreiber, 1993), β -chain expression is restricted to a small number of cells that are responsive to IFN- γ (Gajewski and Fitch, 1988). Expression of IFN- γ R α -chain thus constitutes a possible mechanism for clearance of overproduced IFN- γ (Farrar and Schreiber, 1993). Interaction with TCR or CD3 of T

lymphocytes leads to upregulation of β -chain expression (Sakatsume and Finbloom, 1996). In contrast, IFN- γ itself appears to be able to downregulate the expression of the receptor β -chain, thereby limiting the ability of certain cell types to respond to further exposure to IFN- γ (Bach *et al*, 1995).

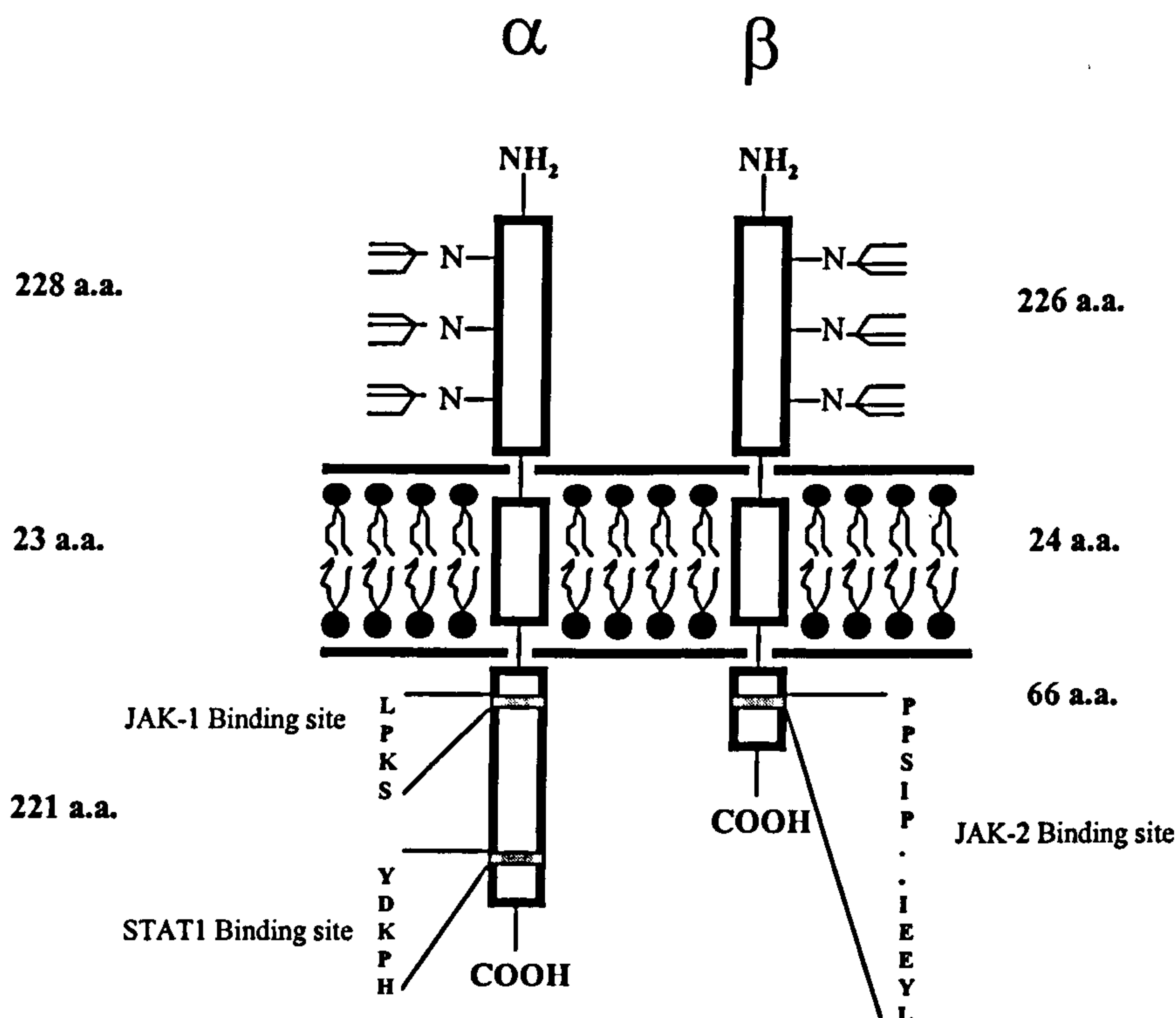


Figure 1.8 Structure of the human IFN- γ R. Both α and β subunits are divided in extracellular, transmembrane and intracellular domains. Numbers relate to size (in residues) of the distinct domains. Functionally important sequences of both subunits are represented. (Bach *et al*, 1997).

The polypeptide chains of the IFN- γ R (Figure 1.8.) are organized in a similar fashion and belong to the class II cytokine receptor family (Bazan, 1990). In the human and mouse, the α -chain comprises an intracellular domain (of 221 and 200 a.a. respectively), a transmembrane domain (of 23 a.a.) and a extracellular domain of (228 a.a.) It has a molecular weight of 90 Kda under denaturing conditions. Similarly the β -chain (60 to 67

Kda) has an extracellular domain of 226 a.a. in the human and 224 a.a. in the mouse, a transmembrane domain of 24 a.a. and an intracellular domain of 66 a.a. (Hibino *et al*, 1991; Cook *et al*, 1994).

Within the intracellular domain of the α -chain there are three functionally important motifs: the first, Leu-Ile (residues 270-271), plays a role in directing receptor trafficking through the cell; the second, $_{266}$ Leu-Pro-Lys-Ser $_{269}$, binds the tyrosine kinase Jak1; finally, the motif $_{440}$ Tyr-Asp-Lys-Pro-His $_{444}$ binds STAT 1, where the tyrosine in position 440 is responsible for phosphorylation of STAT1. Similarly in the β -chain intracellular domain the motifs $_{263}$ Pro-Pro-Ser-Ile-Pro $_{267}$ and $_{270}$ Ile-Glu-Glu-Tyr-Leu $_{274}$ (Bach *et al*, 1997) bind Jak2. The overall identity between human and mouse of the α and β polypeptide chains is 52.5 and 58%, respectively. However the intracellular domain of the β -chain of the mouse shares 78% of homology with its human counterpart. Experiments employing human:mouse chimeric receptors revealed that IFN- γ R α -chain and β -chain extracellular domains must match the host species of IFN- γ for the development of biological activity (Hibino *et al*, 1992; Hemmi *et al*, 1994). Crystallographic studies (Walter *et al*, 1995) and the use of cells expressing either α or β -chain (Marsters *et al*, 1995) showed that the β -chain stabilizes the receptor complex increasing binding of IFN- γ fourfold. These studies also elucidated the general organisation of the receptor and structural changes resulting from the interaction of the IFN- γ R with its ligand (figures 1.8 and 1.9).

After signalling, the IFN- γ : α -chain complex is internalized and dissociated. Free IFN- γ is trafficked to the lysosome where it is degraded. Uncoupled receptor α -chains enter a large intracellular pool of receptors and are eventually recycled back to the cell surface. Crucially the receptor β -chains are not recycled and *de novo* expression of these chains is therefore required for further receptor activity (Bach *et al*, 1997).

1.7.4 Signal Transduction Pathway

IFN- γ signals through the Jak-STAT pathway in a process common to most cytokines (reviewed in 1.4). The IFN- γ signalling pathway is reviewed in Bach *et al* (1997) and a graphic representation of the process is shown in figure 1.9.

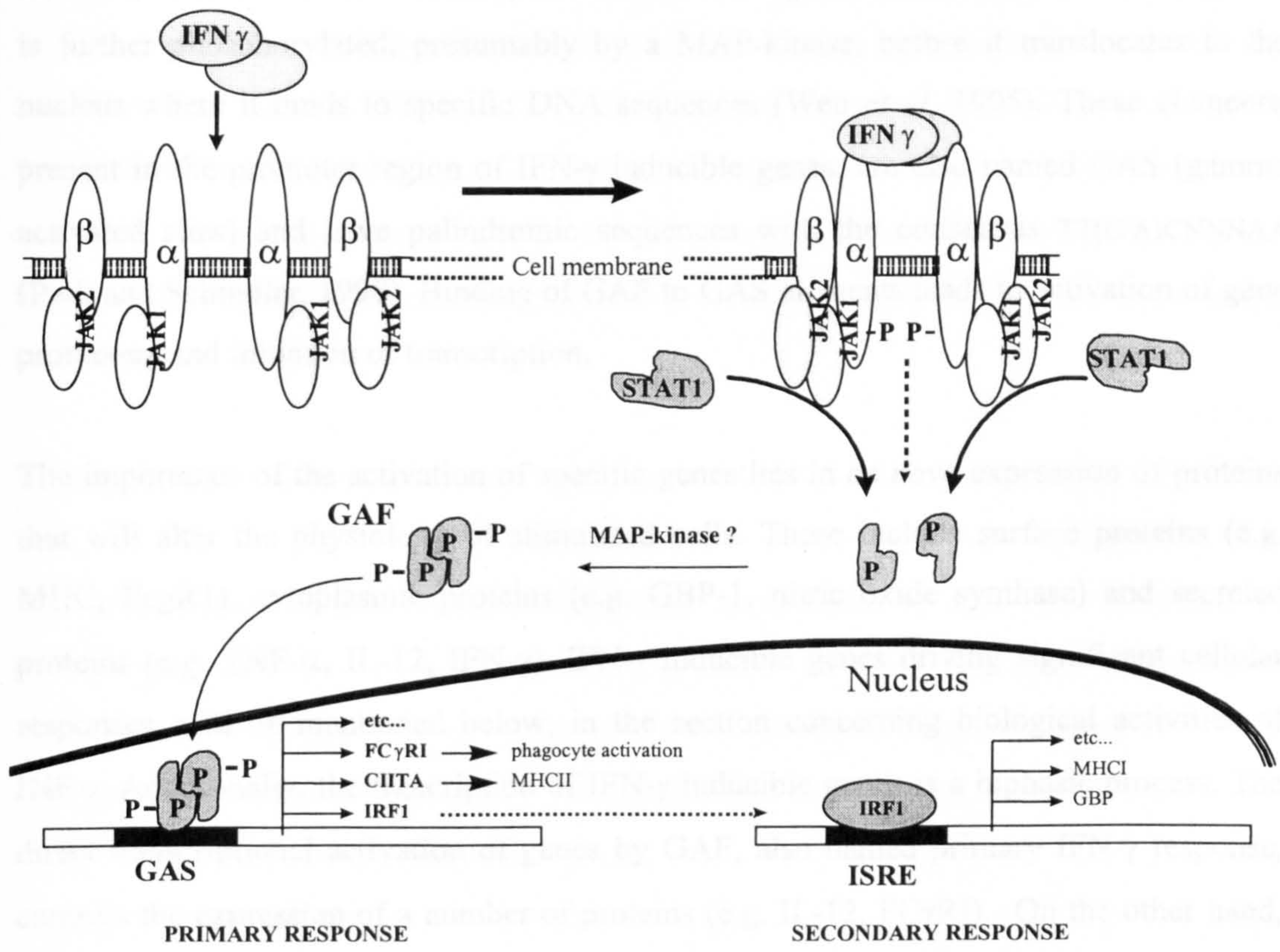


Figure 1.9 JAK-STAT signalling pathway in response to human IFN- γ . Illustrating oligomerisation of the receptor complex; phosphorylation and dimerisation of STAT1; translocation of the STAT1 complex (GAF) to the nucleus and activation of transcription of genes containing GAS and ISRE sequences (modified from Bach *et al*, 1997; Park and Shindler, 1998).

In unstimulated cells, the IFN- γ R α and β subunits are dissociated, binding through their intracellular domain to JAK1 and JAK2 respectively. Binding of the IFN- γ homodimer to the receptor leads to dimerisation of two α -chains, generating a site recognised by the receptor β -chains as they slide towards the α -chains in the centre (Kotenko *et al*, 1995).

Assembly of the receptor brings JAK1 and JAK2 into close association, promoting transphosphorylation and reciprocal activation of the JAKs. The activated JAKs phosphorylate tyrosine residue 440 of the α -chains, generating two binding sites for STAT1. Binding of latent cytoplasmic STAT1 to the activated α -chains leads to phosphorylation, dissociation from the receptor and dimerisation of STAT1 (Greenlund *et al*, 1994). The STAT1 homodimer, also named GAF (gamma-interferon activation factor) is further phosphorylated, presumably by a MAP-kinase, before it translocates to the nucleus where it binds to specific DNA sequences (Wen *et al*, 1995). These elements, present in the promoter region of IFN- γ inducible genes, are also named GAS (gamma activated sites) and have palindromic sequences with the consensus TT(C/A)CNNNAA (Park and Schindler, 1998). Binding of GAF to GAS elements leads to activation of gene promoters and initiation of transcription.

The importance of the activation of specific genes lies in *de novo* expression of proteins that will alter the physiology of stimulated cells. These include surface proteins (e.g. MHC, Fc γ R1), cytoplasmic proteins (e.g. GBP-1, nitric oxide synthase) and secreted proteins (e.g. TNF- α , IL-12, IFN- γ). IFN- γ inducible genes driving significant cellular responses, will be mentioned below, in the section concerning biological activities of IFN- γ . Additionally, the transcription of IFN- γ inducible genes is a biphasic process. The direct transcriptional activation of genes by GAF, also named primary IFN- γ response, controls the expression of a number of proteins (e.g. IL-12, FC γ RI). On the other hand, GAF also initiates the synthesis of a number of secondary transcription factors (e.g. CIITA, p48, IRF-1) that will in turn direct protein expression, often mediating overlapping activities with other cytokines. For example, the class II transactivator (CIITA) is a transcriptional factor required for the expression of MHC class II molecules. Interferon regulatory factor-1 (IRF-1) and p48 play a complex role in regulating the expression of secondary response genes through promoters containing interferon stimulated response elements (ISRE) with the prototypic sequence AGTTTCNNTTTCNC/T. They initiate transcription of a number of genes (e.g. MHC class I, GBP-1, IFN- β) that overlap IFN- α activity (reviewed in Bach *et al*, 1997; Gallin, 1995; Park and Schindler,

1998). Although the role of the Jak-STAT pathway in mediating IFN- γ activity is indisputable, evidence is gathering showing that at least some activities of IFN- γ may be mediated not by the JAK-STAT pathway, but by alternative pathways involving phosphorylation of PKC and mobilisation of Ca^{2+} (Smith *et al*, 1990)

The restricted number of transcriptional factors (e.g. STATs) available for signalling, has a bearing on the specificity of cytokine activity (reviewed in 1.4). In fact, a number of cytokines (e.g. IFN- α , TNF- α , IL-1, IL-2, IL-12) show at least some overlapping functions with IFN- γ , highlighting the redundancy of cytokine activity (Gallin, 1995). Therefore IFN- γ actions should not be viewed as consequences of IFN- γ acting alone but rather in the context of an immune response involving multiple cell-surface and secreted molecules.

1.7.5 Biological Activities

The mechanisms of host defence unravelled in response to IFN- γ are mediated by proteins produced by stimulated cells. These proteins can either alter the metabolic status of the cell or be responsible for more specialised activities. Below is a brief review on cellular changes induced by IFN- γ stimulation and their impact on the regulation of immune and inflammatory responses .

Biochemical changes in response to IFN- γ

Among the biochemical changes experienced by cells upon stimulation with IFN- γ are those that trigger alterations in cellular metabolic pathways involved in the cellular response to pathogens.

TRYPTOPHAN METABOLISM

The proteins indoleamine-2-3-dioxygenase (IDO) and tryptophanyl-tRNA synthetase (WRS) are two enzymes involved in the tryptophan metabolism that are strongly induced by IFN- γ . IDO is the first enzyme in the kynurenine pathway that transforms tryptophan

into alanine and metabolites (e.g. acetyl CoA, quinolinic acid) and is the main contributor to the decrease in serum tryptophan in individuals exposed to IFN- γ . Because tryptophan depletion leads to an overall reduction of protein synthesis, IDO has been implicated in the antiproliferative role of IFN- γ . There is speculation that induction of WRS may safeguard the synthesis of tryptophan-rich, immunologically relevant molecules (e.g. IRF family of proteins) in face of IDO-mediated depletion of tryptophan (reviewed in Burke *et al*, 1995).

RESPIRATORY BURST

IFN- γ potentiates the production of highly reactive oxidants [e.g. hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot-}$)] in activated macrophages and neutrophils. This process is named respiratory burst and is a characteristic of the microbicidal state of macrophages. IFN- γ is directly responsible for the activation of transcription of genes coding for membrane-associated enzymes of the NADPH oxidase system that promote this effect (reviewed in Cassatella *et al*, 1990).

GENERATION OF NITRIC OXIDE

Nitric oxide (NO) is an intercellular messenger that participates in various cellular processes (e.g. vascular homeostasis, neurotransmission, antimicrobial and antiviral defense). NO is formed from L-arginine and molecular oxygen in a reaction catalysed by the enzyme nitric oxide synthase (iNOS), expressed in most cell types upon appropriate stimulation. IFN- γ controls the production of NO via activation of gene transcription of both iNOS and its cofactor tetrahydrobiopterin. However maximal activation of cells to produce NO *in vitro* is only obtained by combination of IFN- γ and endotoxin, IFN- γ and TNF- α or IFN- γ and IL-1. Induction of NO production in phagocytic cells is associated with reduced survival of ingested microorganisms (e.g. *Mycobacteria*, *Toxoplasma*, *Trypanosoma*, *Leishmania*) and is also implicated in the antiviral effect of IFN- γ in macrophages infected with vaccinia and herpes simplex viruses. Additionally, NO secreted by activated macrophages has been associated with tumour cell killing. In this case, the cytotoxic effect of induced NO, may also cause undesirable tissue damage (Moncada and Higgs, 1993; Green *et al*, 1991; Karupiah *et al*, 1993).

Antiviral effects

IFN- γ itself is not a potent antiviral agent when compared to type I IFNs, in fact it has 10-100 fold lower specific antiviral activity *in vitro*, than either IFN- α or IFN- β (Farrar and Schreiber, 1993). Nevertheless, IFN- γ plays an important role in the outcome of viral infections *in vivo*, as documented in a series of experiments including the prophylactic treatment of cytomegalovirus infection in mice through exogenous application of IFN- γ (Fennie *et al*, 1988) an effect neutralised by antibodies against IFN- γ in rats (Haagmans *et al*, 1994) and the increased susceptibility to certain viruses (e.g. vaccinia virus, vesicular stomatitis virus, hepatitis virus) in IFN- γ or IFN- γ R gene knock-out mice (Muller *et al*, 1994; Lucchiari *et al*, 1992).

The overall antiviral activity of IFN- γ can be divided into two interdependent sets of mechanisms. The first entails direct antiviral effects through activation of STAT1 or overlapping with the type I IFN signalling pathway and the second those mechanisms mediated by its immunomodulatory activity. IFN- γ , through phosphorylation of STAT1, directly activates genes containing GAS elements that are involved in the resolution of viral infections. Additionally, IFN- γ stimulation enhances the expression of members of the interferon regulatory factor (IRF) family of transcription factors (e.g. IRF-1, p48, ICSBP-interferon consensus sequence binding protein) and therefore overlaps IFN- α activities. The regulation of secondary response genes by IRFs is a complex and highly regulated process, still largely undisclosed. IRF-1 and p48 are implicated in the activation of gene promoters containing ISRE motifs and of the positive regulatory domain (PRDI) of the IFN- β gene promoter, while ICSBP appears to antagonise their activity, repressing gene transcription. Although IRF-1 has some DNA-binding activity it appears that p48 is required to confer ISRE specificity. ICSBP seems to act through interaction with p48 to inhibit DNA-binding (Kimura *et al*, 1994; Tanaka and Taniguchi, 1992; Bovolenta *et al*, 1994).

IFN- γ can therefore, directly or indirectly, increase the gene transcription of a number of proteins responsible for the antiviral state such as the dsRNA dependent protein kinase PKR, (2'-5') oligoadenylate synthetase, and dsRNA specific adenosine deaminase:

- PKR is a serine-threonine kinase activated by binding to double stranded (ds) RNA structures. Both DNA and RNA viruses produce RNA intermediate products that can activate PKR. Activation leads to auto-phosphorylation, followed by the phosphorylation of the α subunit of eIF2, the eukaryotic protein synthesis initiation factor, thus inhibiting translation and therefore protein synthesis (Hovanessian, 1993). PKR is constitutively expressed in low amounts in many cell types, its promoter contains both ISRE and GAS elements, making it responsive to both type I and II IFNs. Additionally, NFkB elements in the promoter of this gene, reveal overlapping activity in response to TNF- α (Tanaka and Samuel, 1994). The recognition of PKR importance in the antiviral response promoted by IFN- γ came from studies using a model of encephalomyocarditis virus (ECMV) infection in mice. Following treatment with IFN- γ , normal mice had a prolonged survival to the virus, when compared with PKR-deficient mice (Yang *et al*, 1995).

- (2'-5') oligoadenylate synthetase is also constitutively expressed in several cell types and is induced by type I IFNs and less potently by IFN- γ . When activated, the enzyme polymerises ATP into a series of 2'-5' linked oligomers [ppp(A2'p) $_n$ - with four isoforms ($n=1$ to $n=4$)], which in turn activate the latent cellular endoribonuclease (Rnase L) which is responsible for antiviral activity through single stranded RNA degradation. However, degradation is not limited to viral RNA as cellular ribosomal and mRNA are also degraded in the process (reviewed in Hovanessian, 1991; De Maeyer and De Maeyer-Guinard, 1998).

- dsRNA specific adenosine deaminase (dsRDA) is an enzyme that catalyses the deamination of adenosine into inosine using dsRNA as substrate, producing so called edited mRNAs whose translation does not lead to functional proteins. Exposure of cells to both IFN- α and IFN- γ significantly increases transcription of dsRDA (Patterson *et al*, 1995).

The second set of mechanisms governing recovery from viral infection is mediated by immune responses largely orchestrated by IFN- γ . Among the actions that skew the immune response towards a cell mediated state are, as described below, activation of NK cells and macrophages, antibody class switching from IgG1/IgE to IgG2/IgG3, promotion of Th1 over Th2 type responses, maturation of CTLs and upregulation of MHC class I and II presentation pathways. Because NK cells and T cells produce IFN- γ , a positive feedback loop is created that amplifies local cell killing. Paradoxically IFN- γ appears to, at least *in vitro*, render target cells refractory to NK cell activity although retaining susceptibility to CTL killing (Wallach, 1983). These observations lead to the assumption that IFN- γ might increase the development of specific immune responses elicited by CTLs in detriment of the unspecific cell killing mediated by NK cells, as the immunological process progresses (Reiter *et al*, 1991).

Striking evidence of the relevance of IFN- γ expression as a defence mechanism against viral infections comes from observations that some viruses evolving under immunological pressure have acquired genetic codes, by recombination with host DNA, that specifically evade IFN- γ activity. Examples of these are the expression of soluble receptors for IFN- γ encoded by poxviruses that serve as a decoy for IFN- γ binding (Alcani and Smith, 1995) and the production of IL-10-like protein, a potent antagonist of IFN- γ activity, encoded by the human herpes virus, Epstein-Barr (Sairenji *et al*, 1998).

Antigen presentation

The recognition of foreign antigens is a critical step in the development of specific immune responses. Because T cells are unable to recognize antigens in free or soluble form, these have to be presented to them as portions of protein antigens (peptides) that are associated, non-covalently, with major histocompatibility complex (MHC) molecules. The MHC is a large genetic region of human chromosome 6, coding for both class I and class II determinants, as well as other proteins involved in processing and transport of antigens. Both classes of MHC are structurally similar with a highly polymorphic extracellular

peptide binding domain, an extracellular Ig-like domain, transmembrane and cytoplasmic domains. The size of the binding domains, in both classes, restrict the size of the peptides that can be presented (9 to 11 residues in the case of class I and up to 30 amino acids in class II), meaning that foreign proteins have to be processed prior to loading and presentation on the cell surface. MHC molecules bind only one peptide at a time and although this association is one of low affinity, the complexes once formed persist for sufficiently long time to be recognized by T cells (reviewed in Germain, 1994; Abbas⁵ *et al*, 1997). In table 1.7 are presented some differential features of Class I and II antigen processing and presentation pathways.

Table 1.7 Comparative features of MHC class I and II presentation pathways (Abbas⁵ *et al*, 1997).

Feature	MHC class I	MHC class II
Composition of MHC complex	polymorphic α -chain, β_2 microglobulin, peptide	polymorphic α and β chains, peptide
Type of APC's	all nucleated cells	mononuclear phagocytes, B lymphocytes, dendritic cells, endothelial cells, thymic epithelium
Source of protein antigens	mostly synthesized on polyribosomes in the cell	mostly of extracellular origin
Site of peptide generation	cytosolic proteasome	endosome/lysosome
Site of MHC peptide loading	ER	specialized endosomal/lysosomal compartment
Associated proteins prior to loading	Calnexin, BiP, TAP	Calnexin, invariant chain
Peptide transport for loading	TAP	not necessary
Transport to cell surface	secretory pathway	? secretory pathway ?
Responsive T cells	CD8 ⁺ T cells (CTL's)	CD4 ⁺ T cells (mostly T helper cells)

APC, antigen presenting cell; ER, endoplasmic reticulum; BiP, binding protein; TAP, transporter associated with antigen processing; CTL, cytotoxic T lymphocyte.

The upregulation of MHC molecules was one of the first biological effects attributed to IFNs. IFN- γ not only shares the ability of regulating expression of MHC class I with type I IFNs, but it also regulates elevation of MHC class II presentation (Rosa *et al*, 1986).

This process involves the activation of multiple genes, whose products contribute in distinct ways to antigen presentation.

CLASS I ANTIGEN PRESENTATION

MHC Class I molecules contain two separate polypeptide chains: α or heavy chain and a non MHC encoded β chain ($\beta 2$ microglobulin). Expression of class I molecules on the cell surface depends on the post-translational association of α and β chains (reviewed in York and Rock, 1996). MHC Class I is constitutively expressed in most tissues with a relatively high expression in lymphomyeloid cells. Although all IFNs are able to upregulate class I expression, IFN- γ is generally more active than IFN- α or β . *In vivo* administration of IFN- γ results in a disseminated increase of class I expression on many tissues (Watine *et al*, 1990). The responsiveness to IFNs is mediated mainly by an ISRE motif present in the promoter region of class I heavy chain genes. Although induction of IRF-1 by IFN- γ with binding to ISRE is an important factor in transcription, it appears that other mediators are needed for efficient gene activation. Among these is the TNF- α induction of NF κ B and consequent binding to an enhancer, adjacent to the ISRE that contains three NF κ B binding sites (enhancer A). The enhancer A element, plays a central role in inducible expression of class I genes and is the basis of the strong synergism between IFN and TNF- α in the regulation of MHC class I (Johnson and Pober, 1990). Similarly IFNs increase expression of $\beta 2$ -microglobulin in synergism with TNF- α . IFN- γ also upregulates the expression of other proteins involved in processing of antigens for presentation in the cell surface (Anderson *et al*, 1994; Rotem-Yehudar *et al*, 1996). These include: the proteasome, a multiple subunit endopeptidase responsible for the generation of peptides from foreign proteins; the TAP family of proteins, involved in the transport of peptides to the ER for loading and gp96, a stress protein present in the lumen of the ER and involved in the protection of peptides from degradation or default secretion (reviewed in York and Rock, 1996).

CLASS II ANTIGEN PRESENTATION

The MHC class II presentation pathway is constitutively active only in professional antigen presenting cells (e.g. macrophages, dendritic cells, B cells) and is strongly

upregulated by IFN- γ (Mach *et al*, 1996). Paradoxically while IFN- γ acts to increase class II expression in most cell types it inhibits this antigen presentation pathway in B cells (Mond *et al*, 1986). IFN- γ can also induce *de novo* expression of class II molecules in most cell types, converting the responding cells into non professional antigen presenting cells. These cells, being unable to express costimulatory signals (e.g. B7, ICAM-1) are thought to play an important role in the induction of T cell anergy as a mechanism of immunological tolerance (Lenschow, 1996). B7 and ICAM-1 expression is also enhanced by IFN- γ (Freedman *et al*, 1991). All the key genes required for normal expression of MHC class II at the cell surface are regulated by IFN- γ . These include, α and β chains that form the class II molecule; the ER chaperone Ii, proteins responsible for peptide loading (DMA and DMB) and lysosomal cathepsins. IFN- γ induction of MHC-II is orchestrated by a single transcription factor, the class II transactivator (CIITA) in a highly specialised process that is a paradigm of non redundant cytokine activity. Although, because CIITA itself has no DNA binding activity, is thought to activate genes through the interaction with multiple nuclear transcription factors (e.g. RFX). The CIITA gene promoter contains a GAS element and is therefore directly activated by IFN- γ (reviewed in Chang and Flavell, 1995 and Mach *et al*, 1996).

Immunomodulatory activities

EFFECTS ON MACROPHAGES AND NK CELLS

The activation of macrophages as a mechanism of non-specific cell mediated host defence is a well established phenomenon that constitutes one of the major manifestations of cellular response to IFN- γ . This activity, firstly demonstrated *in vitro* in response to supernatants from stimulated T lymphocytes, was given the name of macrophage activating factor and was ultimately shown to be largely accounted for by IFN- γ (Nathan *et al*, 1983). Macrophage activation by IFN- γ entails an increase in the phagocytosis and endocytosis capability of these cells. Phagocytosis is further enhanced by IFN- γ induced expression of the high affinity receptor Fc γ RI that binds to specific IgG molecules on the surface of opsonised bacteria. Once inside macrophages, microorganisms are killed by

toxic molecules, namely reactive oxygen and nitric oxide whose production is also primarily upregulated by IFN- γ (Nathan, 1992). IFN- γ is therefore sufficient to fully activate macrophages for killing of microorganisms. Similarly, in NK cells, IFN- γ upregulates the expression of the low affinity receptor Fc γ RIII and promotes an increase in cytotoxic activity (Farrar and Schreiber, 1993). The upregulation of Fc γ RI, in macrophages, and Fc γ RIII in NK cells increases the capability of these cells to kill neoplastic and virus infected cells by means of antibody dependent cell cytotoxicity (ADCC) (Ravetch and Kinet, 1991). Expression of cytokines (e.g. IL-12, TNF- α) by macrophages and NK cells in response to IFN- γ leads to implementation of synergism and local activation positive feedbacks, contributing for clearance of infection. Additionally, by upregulating the expression of co-stimulatory adhesion molecules (e.g. ICAM-1) in target cells and their ligands (e.g. Mac-1) in macrophages and NK cells, IFN- γ intensifies the killing of virus infected and tumour cells (Bevilacqua, 1993).

EFFECTS ON T LYMPHOCYTES

IFN- γ stimulates proliferation of mitogen-triggered primary T cells and mixed lymphocyte populations and of a variety of T cell lines in contrast to the antiproliferative effect that it exerts in most other cell types. This apparently paradoxical situation is thought to be due to upregulation of both IL-2 and IL-2 receptor in responsive T lymphocytes by IFN- γ (Landolfo *et al*, 1988). IFN- γ also increases the development of CTL activity in antigen-stimulated lymphocyte cultures (Siegel, 1988). This effect results from stimulatory effects on mononuclear phagocytes, T helper cells or CD8⁺ precursors or alternatively from inhibitory effects on suppressor T cells. Additionally, IFN- γ both accelerates and enhances IL-12 determined Th1 phenotype development, possibly by upregulating IL-12 receptor expression on naïve CD4⁺ T cells, in synergism with TCR cross-linking (Wenner *et al*, 1996).

EFFECTS ON B LYMPHOCYTES

Cytokines regulate the switching of heavy chain class in antibody secreting B cells from IgM to other downstream isotypes (e.g. IgG, IgE) by activation of transcription of the target isotype genes. IFN- γ is the main switch regulating IgG2a and, to a lesser scale,

IgG3 switching in the mouse. IFN- γ also strongly antagonises IL-4 dependent switching to IgG1 and IgE heavy chain classes. Confirmation of this phenomenon was obtained when treatment of mice with neutralising antibodies to IFN- γ followed by vaccination with influenza virus antigens, resulted in increased levels of antigen specific IgG1 and IgE and reduced levels of IgG2 and IgG3 (Dobber *et al*, 1995). This effect, exerted directly on B cells through activation of STAT1, is dependent on B cell activation and IL-2 stimulation and is enhanced by IL-1 (Snapper *et al*, 1996). There are also reports, although somewhat contradictory, linking IFN- γ with B cell maturation from resting splenic B cells and apoptosis of differentiated B cell lines (Sidman *et al*, 1984; Trubiani *et al*, 1994).

CYTOKINE NETWORK

IFN regulates the expression of a number of cytokines and growth factors or their receptors (see table 1.8) including its own expression and that of its receptor (Farrar and Schreiber, 1993). Receptor autoregulation is an aberrant phenomenon only observed in CD4⁺ T cells. It entails the induction or repression of IFN- γ R β -chain synthesis in Th2 and Th1 cells, respectively (Bach *et al*, 1995). As mentioned before, although β -chains do not take part in IFN- γ binding, they do intervene in signal transduction.

Although several cytokines can reinforce the production and/or activity of IFN- γ (e.g. IL-12, IL-18, type I interferons, IL-1, IL-2), IFN- γ synergises mainly with TNF- α . This synergism is often additive and is observed both *in vitro* (e.g. induction of expression of cell surface molecules, microbicidal activity of macrophages, cytotoxicity of tumour cells) and *in vivo* (e.g. antitumour effects, induction of cytokines, systemic toxicity). The synergism between IFN- γ and TNF- α is largely mediated by overlapping of the signal transduction pathways involving IRF-1 and NF κ B, respectively (Drew *et al*, 1995) and is supported by the strong upregulation of both TNF- α protein and receptor by IFN- γ (see table 1.8).

Table 1.8 Cytokine modulation by IFN- γ .

Cytokine	Response to IFN- γ	Relevant functions
Interleukin-12 (IL-12)	Induction of p40 subunit in macrophages, dendritic cells and other APCs; greatly enhanced by bacterial products (e.g. LPS).	Strong inducer of IFN- γ in TH1, CD8+ and NK cells (1)
Interleukin-18 (IL-18)	Increased secretion by upregulation of ICE.	Synergism with IL-12 in IFN- γ production (2).
Interferon- γ (IFN- γ)	Increased production in peripheral lymphocytes, independent of IL-12.	—
Interleukin-4 receptor (IL-4R)	Contradictory data: upregulation in murine macrophage cell line; downregulation in human PBL.	Antagonist of IFN- γ immune modulation (3, 4).
Tumour Necrosis Factor- α (TNF- α)	Enhancement of production in response to LPS in macrophages.	Synergism with IFN- γ in antiviral and antitumour activity (5).
TNF- α receptor (TNF- α R)	Upregulation of both receptor chains in endothelial, epithelial and myeloid cells.	—
Interferon- β (IFN β)	Upregulation through IRF-1 activation in most cell types.	Synergism with IFN- γ in antiviral and antiproliferative activity (6).
Interleukin-6 (IL-6)	Enhances production in response to IL-1 α in human thymic epithelial cells and murine B cells.	Growth factor for activated B cells (used in hybridoma cell culture); costimulator of T cells (7).
Granulocyte Colony Stimulating Factor (G-CSF)	Represses IL-1 α induced production in human thymic epithelial cells.	Haemapoietic growth factor, promoting maturation, differentiation and activation of neutrophils (7).
Macrophage Colony Stimulating Factor (M-CSF / CSF-1)	Upregulation in synergism with IL-1 α and TNF- α in human thymic epithelial cells.	Haemapoietic growth factor, modulating differentiation, proliferation and activation of mononuclear phagocytes (8)
Stem Cell Factor (SCF) c-kit ligand	Upregulation in synergism with IL-1 and TNF- α in murine bone marrow cell line.	Early haematopoietic factor promoting sensitisation of progenitor cells to colony stimulating factors; increases immature T cell viability (9).

ICE - Interleukin-1 β -converting enzyme
Numbers refer to references: 1- Yoshida et al, 1994; 2- White, 1996; 3- Feldman and Finbloom, 1990; 4- Byron et al, 1991; 5- Koerner et al, 1987; 6- Fujita, 1988; 7- Galy and Spits, 1991; 8- Galy et al, 1993; 9- Gautam, 1995.

Several cytokines have, conversely, an antagonistic effect towards IFN- γ activity and production, representing mechanisms that prevent deleterious effects of continuous IFN- γ production. IL-4, produced mainly by B cells and T helper type 2 cells, is the main antagonist of IFN- γ , countering the majority of its biological activities. Examples of opposing effects of IL-4 on IFN- γ activity are the inhibition of macrophage activation and microbicidal activity and antiviral activity, the induction of cytokines and thymocyte proliferation and the antibody class switching favouring IgG1 and IgE isotypes (Paul, 1991). Nevertheless, the single most emphatic way by which IL-4 antagonises IFN- γ is the skewing of T helper responses towards the Th2 phenotype, therefore directly inhibiting IFN- γ production (reviewed in 1.4).

Other cytokines with antagonistic effects over IFN- γ include:

- TGF- β 1, secreted by a variety of cells (e.g. platelets, fibroblasts, lymphocytes, macrophages), antagonises IFN- γ by inhibiting T lymphocyte proliferation and generation of CTLs, IFN- γ driven MHC II expression and generation of nitric oxide by activated macrophages (Chantry *et al*, 1989);
- IL-10, produced by Th2, monocytic and B cells, directly inhibits the synthesis of IFN- γ in all cell types. Since this antagonism is reciprocal, the balance between IL-10 and IFN- γ appears to be important in the regulation of the course of an immune response (Moore *et al*, 1993);
- IL-13, synthesised mainly by Th2 cells, is structurally related to IL-4, sharing some of its biological activities, including inhibition of IFN- γ driven macrophage activation and microbicidal activity (Zurawski and De Vries, 1994).

IMMUNE SUPPRESSION

IFN- γ has been involved in the induction of “suppressor” (or regulatory) T cells. These cells produce mainly TGF- β 1 and IL-10 and are responsible for the inhibition of the activation phase of immune responses with a consequent decrease in lymphocyte proliferation capability. The T regulatory cell population is characteristically expanded in graft-versus-host disease following bone marrow transplantation (Bloom *et al*, 1992).

This process was found to be, at least in part, regulated by IFN- γ , since treatment of hosts with anti-IFN- γ antibodies relieves symptoms (Wall *et al*, 1988).

The requirement of IFN- γ for appropriate modulation of immune responses is highlighted by the use of IFN- γ or IFN- γ R gene knockout mice. These mice show increased susceptibility to infections by intracellular pathogens due to deficient macrophage activation, reduced production of NO and expression of MHC II by macrophages, reduced serum levels of IgG2a and IgG3 antibodies and defective NK cell function (Abbas⁶ *et al*, 1997).

Effects on Tumour cells

Type I IFNs show an important anti-tumour activity and are currently used as therapeutic agents for patients with a number of different carcinomas. IFN- γ also shows some direct anti-tumoural activity through the production of NO that inhibits tumour growth and the activation of interferon-inducible protein-10 (IP-10) that inhibits angiogenesis and therefore tumour mass growth (Yu *et al*, 1996). IFN- γ also activates indirect effector mechanisms including MHC antigen expression, macrophage activation, stimulation of CTL and NK cell activity (reviewed in De Maeyer and De Maeyer-Guignard, 1998) and has a role in the elicitation of specific tumour-associated antigens (Murray *et al*, 1988). Additionally, IFN- γ exerts a mild antiproliferative effect on most cell types (with the exception of some populations of activated T cells). Moreover, IFN- γ has been shown to bind and activate signal transduction on several neoplastic cell lines and is thought that the downregulation of oncogene (myc) expression might be involved (Kurzrock, 1992).

Role of IFN- γ in infection

Equipped with the information provided in the previous paragraphs we can now draw a general scheme of IFN- γ mediated activity in response to infection. Some pathogens, including bacteria (e.g. *Listeria*, *Salmonella*, *Mycobacterium*) and parasites (e.g. Protozoa, *Chlamydia*, Fungi) use the intracellular environment as a means to escape host

defence mechanisms (e.g. elicitation of microbicidal antibodies, activation of complement). Therefore, clearance of these infections is dependent on cell mediated immune responses. Early in infection, IFN- γ produced mainly by NK cells is responsible for monocyte/macrophage activation and increased phagocytosis capability. IFN- γ also promotes induction of cell surface presentation of pathogen antigens in the context of MHC class II, which leads to activation of CD4⁺ Th1 lymphocytes through TCR cross linking. Th1 cells produce increased levels of IFN- γ that optimises phagocytosis of opsonised pathogens (mediated by Fc γ RI upregulation) and induces microbicidal mechanisms (e.g. reactive oxygen and nitric oxide) of infected phagocytes. A positive feedback loop of cell activation is maintained between macrophages (producing IL-12 and IL-18) and Th1 cells (producing IFN- γ) that leads to the eventual clearance of infection. This cytokine dependent pathway is nonetheless complemented by cytotoxic T cell activity that specifically kills phagocytes or other infected cells, presenting pathogen peptides in the context of MHC class I. As described previously, both CTL activation and MHC class I expression are actively induced by IFN- γ .

IFN- γ is instrumental in host defence against viral infection, with functions both in the elimination of virus following primary infection and in the establishment of appropriate immunity against reinfection. Whether the *in vivo* antiviral effects of IFN- γ are due to its direct induction of antiviral proteins (e.g. PKR, dsRDA) or to its immunomodulatory activities (e.g. activation of NK cells and ADCC, maturation of CTLs, regulation of Th1 in detriment of Th2 type responses) is a subject still in debate.

The role of IFN- γ in host defence is emphasised when murine IFN- γ or IFN- γ R gene knockout models are challenged with infectious organisms. These mice show several failures in immune function that result in increased susceptibility to intracellular pathogens such as *Leishmania major*, *Listeria monocytogenes*, Mycobacteria and vaccinia virus (reviewed in Farrar and Schreiber, 1993). Nevertheless IFN- γ is not always beneficial for the resolution of infectious diseases and can also act to the detriment of the host. Examples of detrimental activity of IFN- γ include: the onset of cerebral malaria; the

conversion of an inapparent to an aggressive infection in response to lymphocytic choriomeningitis virus; the stimulation of HIV replication in monocytic cells and the contribution to the progression of endotoxin triggered septic shock pathology, either directly or indirectly, through the induction of TNF- α production (reviewed in De Maeyer and De Maeyer-Guignard, 1992; Farrar and Shreiber, 1993).

1.8 Background to the project

Due to the low level of cytokine cross-reactivity between species, investigation of cytokine biology is often dependent on the development of species-specific reagents such as recombinant cytokines and monoclonal and polyclonal antibodies to those cytokines. Advances in the standardisation of molecular biological and immunological techniques have prompted the cloning of a number of equine cytokines following the lead of human immunology and that of rodent models.

Table 1.9 Equine cytokines cloned to date

Equine cytokine	Accession number	Reference
IFN- α 1/2/3/4	M14540 / M14541 M14542 / M14543	Himmler <i>et al</i> , 1986
IFN- β	M14546	Himmler <i>et al</i> , 1986
IFN- γ	D28520	Curran <i>et al</i> , 1994; Grunig <i>et al</i> , 1994
IFN- ω 1/2	M14544 / M14545	Himmler <i>et al</i> , 1986
IL-1 α	D42146	Kato <i>et al</i> , 1995
IL-1 β	D42147	Kato <i>et al</i> , 1995
IL-1RA (receptor antagonist)	D83714	Kato <i>et al</i> , 1997
IL-2	L06009	Vandergrift and Horohov, 1993
IL-4	L06010	Vandergrift <i>et al</i> , 1994
IL-5	U91947	Vandergrift and Horohov, 1997 (unpublished)
IL-6	AF041975	Lai, 1998 (unpublished)
IL-10	U38200	Swiderski, <i>et al</i> , 1995 (unpublished)
IL-12 p40 / p35	Y11129 / Y11130	Nicolson <i>et al</i> , 1999 (in press)
IL-18 (IGIF)	Y11131	Nicolson <i>et al</i> , 1999 (in press)
TGF β -1	X99438	Penha Goncalves <i>et al</i> , 1997
TNF- α	M64087	Su <i>et al</i> , 1993 (unpublished)

Accession number- for access to the nucleotide sequence and putative amino acid sequence of genes or cDNAs in GeneBank.

However, when this project was initiated very few equine cytokine genes had been sequenced and equine IFN- γ cDNA had just been cloned. The cloning of equine Th1 cytokines was therefore an essential step in the fulfilment of the aims of this project that were to characterise recombinant equine IFN- γ and to determine whether this cytokine, and cytokines inducing IFN- γ expression, had application as viral vaccine adjuvants.

Following the trend in modern medicine for the application of cytokine and anti-cytokine therapies (e.g. soluble cytokine receptors, anti-cytokine antibodies) so too the production of recombinant equine cytokines and their receptors will facilitate their use in therapeutical or prophylactic regimens as well as in vaccine adjuvancy (Nicolson *et al*, 1993). For the sake of completeness an up to date list of equine cytokines sequences is presented in table 1.9.

CHAPTER 2

MATERIALS AND METHODS

The contents of this chapter will be referred to as “mat&meth” in the remaining of this thesis. In the present chapter will only be enumerated materials and methods of general use. Specific procedures and corresponding reagents and equipment will be detailed in the appropriate section of each chapter.

2.1 Materials

2.1.1 Major equipment

- Automated Processor Kodak X-omat processor ME-3 (Eastman Kodak Co., USA).
- Automatic Sequencing Apparatus and appliances: Licor 4000, Licor Inc., USA.
- Cell freezer: Model Kryo10 series II (Planer Products Ltd., UK)

CENTRIFUGES:

- Microfuge 5415C (Eppendorf GMBH, Germany).
- Benchtop centrifuge GS-6R (Beckman Instruments Inc., USA).
- Ultracentrifuge J2-21(Beckman Instruments Inc., USA)
- Fluorescence Assisted Cell Sorter (FACS): Epics Elite (Coulter corporation, USA).
- Gel Documentation System: (Ultra Violet Products Inc., USA).
- Gel drier: Model 583 Gel Dryer (Bio-Rad, USA)
- Incubators for tissue culture (Heraeus Sepatech and Leec Ltd., UK).
- Pipetteman (P20, P100, P200, P1000): (Gilson Medical Electronics, France).
- Spectrophotometer Model DU640, Beckman.
- Thermal Cycler: GeneAmp 9600 (The Perkin Elmer Corporation, USA).
- Vacuum dessicator: Hetovac, Heto Laboratory Equipment, Denmark.
- Water baths (Grant Instruments Ltd., UK).

2.1.2 Consumables

- Acrodisc syringe filters 0.22 and 0.4 μm (Gelman Sciences, USA).
- Bottle top filters 0.22 μm pore size (Sigma, UK).
- Cell scrapers (Greiner Labortechnik Ltd, UK)
- Disposable, sterile scalpels (Swann-Morton, UK).

- Falcon Tubes 15 and 50ml (Becton Dickinson labware, UK).
- Filter tip pipette tips 30 μ l and 200 μ l (Rainin Instrument Co., USA).
- Flat ended gel loading tips (Sorenson Bioscience Ltd., Denmark).
- Cryo-tubes (2ml) Nunc (Nalge Nunc International, Denmark)
- PCR tubes (The Perkin Elmer Corporation, USA).
- Petri dishes, bijoux and universals (Greiner).
- Pipette tips yellow (200 μ l) and blue (1000 μ l) (Sarstedt).
- Screw top and flip top 1.5 ml eppendorf and 0.5 ml tubes (Treff AG, Switzerland).
- Syringes of 2, 5, 10, 20 and 50 ml (Becton Dickinson labware, UK)
- Tissue culture disposables: Costar Flasks, Multiwell plates, Pipettes (Corning Inc., USA).

2.1.3 Chemicals

All general chemicals used were of analytical (Analar) or ultra pure grade and were supplied by Sigma Chemical Company (Dorset, UK) or BDH Ltd. (Poole, UK) unless otherwise stated.

Radiochemicals: ($\alpha^{32}\text{P}$)-dCTP Redivue (specific activity 3000Ci/mmol) and methyl- ^3H Thymidine (specific activity 74 GBq/mmol) were supplied by Amersham Life Science (Bucks, UK).

2.1.4 Complete kits

- ELISA Starter Kit supplied by Pierce & Warriner (Chester, UK) for evaluation of antipeptide equine serum.
- Gene Amp PCR Core Reagents (Roche Molecular Systems Inc., USA) supplied by Applied Biosystems Ltd.
- Mammalian Transfection Kit supplied by Stratagene (Amsterdam, The Netherlands) for the transient transfection of COS 7 cells.
- High Prime DNA Labelling Kit (random primed) supplied by Boehringer Mannheim for the labelling of cDNA probes to use in northern blots.
- PCR-Script Cloning Kit supplied by Stratagene (Amsterdam, The Netherlands) for the cloning of blunt end PCR products generated by Pfu polymerase.

- Qiaprep Miniprep System including vacuum manifold supplied by Qiagen (West Sussex, UK) for the isolation of up to 20µg of high-purity plasmid DNA from bacteria.
- Qiagen Plasmid Mega Kit supplied by Qiagen (West Sussex, UK) for the isolation of up to 2.5mg of high-purity plasmid DNA from bacteria.
- QuickPrep mRNA purification Kit supplied by Pharmacia Biotech (Herts, UK).
First Strand cDNA Synthesis Kit supplied by Pharmacia Biotech (Herts, UK).
- Rneasy Total RNA system supplied by Qiagen (West Sussex, UK) for the isolation of total RNA from transfected cells.
- TA Cloning Kit supplied by Invitrogen (Groningen, The Netherlands) for the cloning of PCR products with A overhangs, generated by Taq polymerase.
- Thermo Sequenase Cycle Sequencing Kit supplied by Amersham Life Science (Bucks, UK) for the automated sequencing of dsDNA.
- Wizard PCR Prep DNA Purification System supplied by Promega (Madinson, USA) for the purification of DNA fragments from agarose gels.

2.1.5 Molecular size standards

DNA: 1kb DNA Ladder (size range 500bp to 12 kb) and ϕ X174 RF DNA/Hae III fragments (size range 72 to 1353 bp) supplied by Gibco BRL (Paisley, UK).

Protein: Kaleidoscope Prestained Markers wide range (6.7-205 kDa) supplied by Bio-Rad (Herts, UK).

2.1.6 Other specialist reagents

Bio-Rad Protein Assay Reagent supplied by Bio-Rad, for measuring protein concentration by the Bradford procedure.

ECL Western Blotting Reagents supplied by Amersham Life Science.

Lipofectin Reagent supplied by Gibco BRL, for the co-transfection of insect cells with baculovirus.

ENZYMES:

- Restriction enzymes and respective buffers were supplied by New England Biolabs (Herts, UK) or Gibco BRL.

- DNase I supplied by Gibco BRL, for removing DNA from RNA preparations.
- Murine Moloney Virus Reverse Transcriptase Enzyme supplied as part of the cDNA synthesis kit by Pharmacia.
- Pfu DNA polymerase was supplied by Stratagene.
- RNase A supplied by Gibco BRL, for removal of RNA contaminants from crudely purified DNA
- T4 DNA Ligase supplied by Gibco BRL (or Invitrogen and Stratagene as a component of the cloning kits).
- Taq DNA polymerase as part of the Gene Amp PCR kit.
- Thermo Sequenase DNA polymerase supplied as a component of the cycle sequencing kit by Amersham Life Science.

2.1.7 Oligonucleotides

All oligonucleotides were synthesised by the departmental contractor MWG-Biotech and supplied in dH₂O suspension. Sequencing primers were modified by IRD800 labelling. Sequences of the primers are detailed in the appropriate chapters.

2.1.8 DNA Plasmid vectors

- pCDNA3.1 Vector supplied by Invitrogen. Designed for transient or selectable constitutive gene expression in mammalian cells expressing the SV40 large T antigen (e.g. COS-7 cells).
- pCI-neo Mammalian Expression Vector supplied by Promega. Designed for mammalian expression as above.
- pCR®II TA Cloning Vector purchased from Invitrogen as part of the TA cloning kit. Supplied as linearised DNA with 3' T overhangs.
- pCR-Script Cloning Vector purchased from Stratagene. Supplied as blunt end linearised DNA.
- pGEX-4T-1 Vector supplied by Pharmacia Biotech. Designed for inducible intracellular expression in *E. coli* of genes as fusion proteins with *Schistosoma japonicum* glutathione-S-transferase.

2.1.9 Bacterial strains

- *E.coli* DH5 α cells (Gibco BRL): F' ϕ 80*lacZ* Δ M15 Δ (*lacZYA-argF*)U169 *deoR* *recA1* *endA1* *hsdR17*(r_k^- , m_k^+) *supE44* λ^- *thi-1* *gyrA96* *relA1*.
- *E.coli* INV α F' cells (Invitrogen): F' *endA1* *recA1* *hsdR17*(r_k^- , m_k^+) *supE44* *thi-1* *gyrA96* *relA1* ϕ 80*lacZ* Δ M15 Δ (*lacZYA-argF*)U169 *deoR*⁺ λ^- .
- *E.coli* JM105 cells (Pharmacia Biotech): *thi* *rpsL* *endA* *sbcB15* *hsdR4* *SupE* Δ (*lac-proAB*)/F' [*traD36* *roAB*⁺ *LacF*⁺ *LacZ* Δ M15]. Host restriction minus, modification plus.
- *E.coli* MAX Efficiency DH10BTM (Gibco BRL): F' *mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ 80*lacZ* Δ M15 Δ *lacX74* *deoR* *recA1* *endA1* *araD139* Δ (*ara*, *leu*)7697 *galU* *galK* λ^- *rpsL* *nupG*.
- *E.coli* BL21[DE3] (Stratagene): B F' *dcm* *ompT* *hsdS*(r_B^- m_B^-) *gal* λ [DE3].

The BL21 bacterial cells are deficient in Lon and OmpT proteases and are therefore ideal for supporting protein expression (discussed in chapter 3).

The genotypes *endA1* and *hsdR17* give improved quality of miniprep DNA; *recA1* denotes recombination deficiency, recommended for stable episomal replication of high copy number plasmids. The ϕ 80*lacZ* Δ M15 marker, enables blue-white screening by α -complementation of β -galactosidase (X-Gal) encoded by the plasmid DNA.

2.1.10 Eukaryotic cells

PRIMARY CULTURES

- Equine Peripheral Blood Lymphocytes (equine PBLs). Isolated from complete blood by Ficoll centrifugation gradient (detailed in chapter 3).
- Equine enriched T cells. Isolated from equine PBLs by negative selection using a nylon wool column (detailed in chapter 3).
- Equine enriched lung macrophages. Tissue culture flask-adherent cells from lung washes (detailed in chapter 4).

CELL LINES

- Insect cell lines (SF-9 and SF-21) purchased from Invitrogen. Support optimal replication (SF-9) and optimal protein expression (SF-21) when infected with recombinant AcMNPV baculovirus (Vaughn *et al*, 1977) as detailed in chapter 3.
- COS-7 cell line, supplied by European Collection of Cell Cultures (ECACC). Derived from African green monkey kidney cells transformed with the SV40 large T antigen and has a fibroblast morphology (Gluzman, 1981)
- Equine Embryonic Kidney cell line, kindly supplied by Dr Jay Patel of Intervet UK (The Elms, Cambridge). This cell line has an epithelial-like morphology (Edington *et al*, 1984).
- Madin-Darby Bovine Kidney (MDBK) cell line. Supplied by ECACC and has an epithelial-like morphology (Nanni *et al*, 1986).

2.1.11 Media and supplementsFOR BACTERIA

- Luria-Bertani (LB) medium: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride in ddH₂O autoclaved and stored at room temperature.
- LB-agar: LB medium containing 1.5% (w/v) agar.
- SOC Medium: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose.
- 2 X YT: Bacto-tryptone, bacto-yeast extract, NaCl (GibcoBRL). 31g made up in 1litre of ddH₂O, autoclaved and stored at 4°C.
- Ampicillin: 100mg/ml in dH₂O . Filtered through a 0.22µm filter, aliquotted and stored at -20°C. Used at a final concentration of 100µg/ml.

FOR EUKARYOTIC CELLS

All media and supplements were purchased from Gibco BRL.

- Dulbecco's Modified Eagle's Medium (DMEM): with 1000mg/L D-glucose, Sodium Pyruvate and Pyridoxine.
- Minimum Essential Medium with Earl's salts (EMEM):, without L-glutamine.
- RPMI 1640 medium: without L-glutamine.
- TC 100 Insect medium: with L-glutamine, without Sodium bicarbonate.

- Foetal Calf Serum (FCS), TCS Biologicals Ltd., (Buckingham, UK): virus screened, mycoplasma screened. Heat inactivated.
- Gentamicin: supplied as gentamicin sulphate 50mg/ml in dH₂O and used at 10µg/ml.
- L-glutamine: supplied as 200mM (100X) stock solution.
- MEM Non Essential Amino Acids (NEAA): supplied as 100 x solution containing L-alanine (890 mg/L), L-asparagine (1320 mg/L), L-aspartic acid (1330 mg/L), L-glutamic acid (1470 mg/L), glycine (750 mg/L), L-proline (1150 mg/L) and L-serine (1050 mg/L).
- Penicillin/streptomycin: supplied as a 100X stock solution of 10,000 units penicillin and 10,000µg streptomycin per millilitre. Used at a final concentration of 100 units penicillin and 100µg streptomycin per ml (1X Penicillin/Strptomycin).
- Trypsin-EDTA: supplied as 10X liquid, stored at -20 °C. This was diluted 1:10 in sterile PBS prior to use and stored at +4 °C.
- β-Mercaptoethanol (2-ME): supplied as 50mM solution in Dulbecco's PBS.

2.1.12 Reagents and solutions

The following list represents only the most commonly used solutions. Other, more specific reagents, will be listed in the appropriate methods section of each chapter.

WATER

Tissue culture grade distilled water was supplied by Gibco BRL. Ultrapure water (for procedures involving recombinant DNA and protein methods was provided by a Millipore Q50 water purification system (Millipore (UK) Ltd., Watford, UK). Water for preparation of general solutions and media was purified using a Millipore RO10 system. For all procedure involving RNA was used DEPC treated water (ddH₂O was treated with 0.05% diethyl pyrocarbonate (DEPC) overnight at room temperature. To remove any trace of DEPC, the DEPC-H₂O was autoclaved for 30 minutes at 120°C.

GEL LOADING BUFFERS

- DNA: 50% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol, 100mM EDTA in ddH₂O. Stored at room temperature and used at a 1:10 dilution.
- RNA: 50% formamide, 2.2M formaldehyde, 1X MOPS, in ddH₂O.

-Protein: 62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β -mercaptoethanol. dH₂O 3.0 ml, 0.5M Tris-HCl, pH 6.8 1.0 ml, glycerol, 1.6ml, 10% SDS 1.6 ml, β -mercaptoethanol 0.4 ml, 0.5% (w/v) bromophenol blue (in dH₂O) 0.4 ml; stored at 4°C. Sample diluted at least 1:4 with buffer and heated at 100°C for 5 minutes prior to loading gel.

- Denhardt's solution (50X): 1% bovine Serum Albumin (BSA), 1% Ficoll, 1% polyvinyl pyrrolidone in ddH₂O. Aliquoted and stored at -20°C.

- Ethidium bromide: 10mg/ml stock in ddH₂O, working solution at 3mg/ml with dH₂O.

- MOPS buffer (10X): 200mM MOPS pH7.0, 50mM potassium acetate, 10mM EDTA. Stored at 4°C in the dark.

- 1 x PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ (pH 7.3).

- 10 x SDS-PAGE Running Buffer: tris base 60 g, glycine 288 g, SDS 20 g. Made up to 2 L by addition of dH₂O.

- Polyacrylamide solution: Acrylamide/Bis acrylamide stock solution. 30% (w/v) acrylamide, 1.579% (w/v) bis acrylamide, ratio 19:1. Severn Biotech Ltd., stored in the dark at 4°C.

- Phosphate buffered saline (PBS): 100mM NaCl, 80mM di-sodium hydrogen orthophosphate, 20mM sodium di-hydrogen orthophosphate, adjusted to pH7.5. Autoclaved and stored at 4°C.

- Pre-hybridisation buffer Northern blots: 50% deionised formamide, 4 X Denhardt's solution, 4 X SSC, 1.6 X Gene Screen, 0.1% SDS, 8% Dextran Sulphate and 30mg/ml heat denatured, sheared salmon sperm DNA.

- Red blood cells lysis buffer: 1.650g ammonium chloride, 0.20g potassium bicarbonate, 0.0074g EDTA (tetra) adding dH₂O to a volume of 200ml.

- RNase A: Prepared as a 10mg/ml stock in 10mM Tris-HCl pH7.5 and 15mM NaCl. Boiled for 15 minutes and cooled slowly. Stored at -20°C.

- SDS-PAGE Protein Gel Fix-Stain Solution: dH₂O 45 ml, methanol 45 ml, HAc 10 ml, Coomassie Brilliant Blue R250 0.25g. Filtered through Whatman No. 1 filter paper.

- SDS-PAGE Protein Des-Stain Solution: dH₂O 85 ml, methanol 5 ml, HAc 10 ml.

- 10 x Semi Dry Transfer Buffer (Towbin): Tris base (48mM) 58 g, glycine (39mM) 29 g, SDS (0.01%) 1 g. dH₂O to 1L. Working stock was prepared prior to use by the addition of 100 ml 10 x stock to 200 ml methanol and 700 ml dH₂O.
- SSC (20X): 3M NaCl, 0.3M Sodium Citrate in dH₂O and adjusted to pH7.0. Stored at room temperature.
- STET: 8% sucrose, 50mM Tris-HCl pH8, 50mM EDTA pH8, 5% triton X100 made up in ddH₂O, filtered (0.22 mm). Stored at room temperature.
- TAE (50X): 2M Tris-HCl pH8.15, 1.5M NaOAc, 1M NaCl, 0.1M EDTA.
- TBE (10X): 0.9M Tris-HCl, 0.9M Boric acid, 25mM EDTA pH8.3.
- TE: 10mM Tris-HCl, 1mM EDTA adjusted to pH8.0. Autoclaved.
- 10 x Tris Buffered Saline (TBS): Tris base 24.2 g, NaCl 80.0 g, HCl 38.0 ml. pH 7.6. dH₂O to 1L.
- 1M Tris HCl: 121g Tris base, 800ml dH₂O. Adjusted to desired pH with concentrated HCl and made up to 1L.
- X-gal solution: prepared as 40 mg/ml stock in dimethylformamide, stored at -20°C.

2.2 Methods

2.2.1 Eukaryotic cell culture

This section will only refer to established cell lines, as primary cell cultures were of short term (chapters 3 and 4).

MAINTENANCE OF CELLS

COS-7 cells were maintained in DMEM, 10% FCS, 2mM L-glutamine and 1X penicillin/Streptomycin. EEK cells in EMEM, 10% FBS, 2mM glutamine, 1X Pen/Strep. MDBK cells in EMEM, 5% FBS, 2mM glutamine, 1X Non essential amino acids, 1X Pen/Strep. These mammalian cell lines were grown in monolayers and maintained in plastic tissue culture flasks (75cm² with 20ml of medium or 135cm² with 35 ml) in a 37°C atmosphere with 5% CO₂. Cells were subcultured every 1 to 2 times a week depending on rate of bulking (reflected in the seeding density). The subculture routine was performed by removing the culture medium, washing the cells once with PBS (equilibrated at 37°C) followed by the addition of 2ml (small flasks) or 4ml (large flasks) of trypsin-EDTA and incubation at for 37°C 15 to 20min. Two volumes of medium are added to the flasks and the cells are removed and washed by vigorous resuspension and centrifuged at 1000g for 5 min. Cells are resuspended in fresh medium to the required density, distributed by the required number of flasks and incubated as before.

Insect cells were maintained in TC-100 medium, 10% FCS and 10µg/ml gentamycin at 27°C. Cells were subcultured 2 to 3 times a week (as they grew rapidly and showed contact inhibition) by removing the culture medium, adding 10 ml of fresh medium and scrape cells off using a disposable cell scraper. The cells were then diluted to desired density and incubated as before.

STORING AND REVIVING CELLS

For long term storage cells were treated as follows. Mammalian cell lines after trypsinisation were washed, counted and resuspended at 1X10⁷ cells/ml in 90% FCS and 10% dimethyl sulphoxide (DMSO- Analar, BDH Ltd). They were then aliquoted

(1ml) in cryo-tubes and stored overnight in the first shelf of a liquid nitrogen storing rack (the tubes were above the liquid level allowing the cells to freeze slower). Next day cells were transferred to the bottom shelves. When required cells were quickly thawed by immersion of the in warm dH₂O and the contents of a full aliquot transferred to a 135cm² flask containing complete medium pre warmed to 37°C, cells were then maintained as before.

For insect cells the procedure was the same except that the freezing medium was composed of 60% of TC-100 medium 30% of FCS and 10% of DMSO. Also, when recovering cells from frozen, the full contents of one aliquot were transferred to a small (25cm²) flask containing 4 ml of medium at room temperature. They were left to incubate for 1 hour after which the medium was carefully removed and fresh medium added. After 24 hours of incubation the medium was changed once more.

COUNTING CELLS

Cells were counted by trypan blue exclusion of dead cells. A small volume of cell suspension (e.g. 20µl) was dilute with an equal volume of 0.4% trypan blue (Gibco BRL) and 10µl were loaded to a haemocytometer with cover slip. Under the 10X objective of an inverted microscope, 5 of the 9 central fields are counted. The sum of the counted cells is multiplied by 4000 to assign the number of cells per ml.

2.2.2 Molecular biology techniques

Detailed protocols of most of the techniques outlined below can be found in Sambrook et al (1989) and Ausubel et al (1996).

Growth of bacteria

Bacteria were used both for maintenance and replication of plasmid DNA and in one instance, also for production of recombinant protein (chapter 3). Because all the plasmids used contained an ampicillin resistance gene, the addition of ampicillin to all bacterial media, to a final concentration of 100µg/ml (unless otherwise stated), leads to the selective growth of bacteria containing plasmid.

Bacterial cells were stored in glycerol stocks. Bacteria to be revived were streaked on to an LB-agar plate (e.g. 10mm petri dish containing approximately 20ml of LB-agar) using a platinum wire sterilised by naked flame. Plates were incubated overnight in a designated oven at 37°C. Single colonies were picked using a pipette tip and dispersed in 3ml of LB medium (LB broth) in a sterile universal. The inoculated broth was incubated at 37°C overnight in an orbital incubator. Confirmation of the presence of the desired plasmid was attained by DNA minipreparation and restriction analysis (see below). The culture could then be scaled up by dilution in excess of 1/1000 in LB medium and overnight growth. Glycerol stocks were prepared by addition of 200µl of 80% glycerol to 1ml of freshly prepared culture. The mixture was aliquoted (1ml aliquots) and stored at -70°C

Purification of plasmid DNA from bacteria

SMALL SCALE PREPARATIONS

Minipreparations of DNA were used for identification of recombinant plasmids by restriction digestion, following generation of each new construct and also to assess maintenance of the plasmid of interest in recovered bacterial stocks. 'Miniprep' DNA was isolated by the STET method. Briefly, 1.5ml of an exponentially growing overnight culture was pelleted and resuspended in 100µl STET containing 3mg/ml lysozyme and 100µg/ml Lysozyme. The samples were boiled for 45 sec and centrifuged at 14000rpm for 15 min in a microfuge. The DNA was precipitated with 100µl propan-2-ol, pelleted by centrifugation, washed in 70% ethanol, dried and resuspended in 20µl of dH₂O. DNA was stored at -20°C.

High quality small scale plasmid DNA preparations were obtained using the Qiaprep Miniprep System (Qiagen) according to the manufacturers instructions. The DNA obtained in this fashion was used mainly in sequencing reactions but also in intermediate cloning steps of complex cloning strategies. This system of DNA purification is a modification of the classical alkali lysis procedure followed by recovery using a plasmid DNA binding resin. The DNA eluted from the column is

precipitated in an equal volume of propan-2-ol by centrifugation as before, dried and resuspended in 50µl of dH₂O.

LARGE SCALE PREPARATIONS

Preparation of up to 2.5mg high purity plasmid DNA was accomplished using the Qiagen Plasmid Mega Kit in a procedure that is a scale up of the above. DNA recovered by this method was mainly used for transfection of mammalian cells with expression vector chimeras.

Quantitation of nucleic acid concentration

BY SPECTROPHOTOMETRY

For quantitation the sample was diluted 1:100 to a total volume of 500µl in dH₂O. The optical density was measured at 260 nm and 280 nm, in comparison to a blank of dH₂O. The concentration of nucleic acid (N.A.) in the sample was calculated using the formula:

$$[\text{N.A.}] / \mu\text{l} = \text{OD}_{260} \times \text{NAF} \times \text{DF} / 1000,$$

where NAF (nucleic acid factor) is 20 for oligonucleotides, 40 for RNA and 50 for DNA and DF (dilution factor), generally 100 (Sambrook *et al*, 1989). The ratio of the OD readings at 260 nm and 280 nm ($\text{OD}_{260}/\text{OD}_{280}$) was used to estimate the purity of the nucleic acid. Pure preparations of DNA and RNA have an $\text{OD}_{260}/\text{OD}_{280}$ of 1.8 and 2.0, respectively; a lower value suggests possible protein contamination.

BY GEL ELECTROPHORESIS

In cases where it was desired to verify the purity of DNA fragments of a certain size, the concentration of dsDNA was determined by running the sample on a polyacrylamide or agarose gel (see below) and comparing the intensity of the fluorescence of the unknown DNA to that of a known quantity of the appropriate size marker (ϕX174 RF DNA/Hae III fragments or 1kb DNA ladder), following staining with ethidium bromide and visualisation by UV transillumination.

Digestion of DNA with restriction enzymes

Restriction endonucleases were used to digest DNA for analysis and cloning procedures. Typically, 1-2 μ g of DNA was digested in a 20 μ l reaction mix containing the appropriate buffer and 5 to 10 units of the desired restriction enzyme. The reactions were incubated at 37°C for a minimum of one hour. Where the isolation of restriction fragments was required, larger quantities of DNA, generally 5 to 20 μ g, were digested, with the reaction volume and components being increased proportionally. When digesting plasmid DNA prepared by the small scale procedure, likely to contain significant RNA contamination, 10 μ g of RNase A was added to the digestion reaction.

DNA electrophoresis

Depending on the size of the DNA molecules to be separated, gels containing 0.6 to 2% agarose can be used for electrophoresis. In this project most of the gels contained 1.2% agarose, giving good separation of DNA molecules with sizes between 500 and 2000bp. 50 ml to 200ml of gels made in 1X TAE or 1X TBE were poured in perspex trays and wells cast with appropriate combs. Ethidium bromide was added to a final concentration of 0.25 μ g/ml and gels were left at room temperature until set, transferred to perspex tanks, a sufficient appropriate buffer (1XTAE or TBE) was added to submerge the gel and the comb was removed. An appropriate volume of DNA gel loading buffer was added to samples and samples were loaded along with molecular size DNA standards. Gels were run at 50 to 90 volts from 30 to 90 min. Gels were visualised in a short wave UV transilluminator and photographed using a gel documentation system.

Purification of DNA fragments from gels

Following electrophoresis the DNA fragment was excised using a sterile scalpel, under UV illumination. Purification of DNA from the gel slice was accomplished using the Wizard PCR Prep DNA Purification System (Promega) and according to instructions supplied. DNA was eluted in 50 μ l of dH₂O.

Ligation of DNA fragments

Vector and insert DNA were mixed at a molar ratio of 1:5 to 1:10 (typically using 50 to 100 ng vector DNA), with an appropriate volume of 5X ligation buffer and 4 units DNA ligase, in a volume of 10 to 20 μ l. Reactions were allowed to proceed overnight at 14 to 15°C and stored thereafter at -20°C if not used immediately. A control ligation, omitting insert DNA was generally set up in parallel to the above, in order to check for 'background' when performing subsequent bacterial transformations. Ligation of inserts into the plasmids pCR-II (Invitrogen) and pCR-Script (Stratagene) was performed according to the manufacturer instructions.

Transformation of bacteria

The ligation reaction was diluted 1:5 and 1 to 2 μ l was added to 50 μ l of competent *E. coli* cells (see materials), or 1 μ l of TA cloned DNA was added to 50 μ l of INVaF⁺ competent cells (Invitrogen) along with 2 μ l of β -mercaptoethanol. In all cases the mixtures were then incubated on ice for 30 min. Cells were subsequently heat-shocked at 42°C for 45 seconds and returned to ice for 2 min. 450 μ l of SOC medium was then added and incubated with shaking for 60 min. Volumes of 50 and 200 μ l of each transformation were then plated onto LB-agar plates containing 100 μ g/ml ampicillin (and 80 μ g/ml X-gal if plasmids contained lacZ gene or equivalent for blue/white selection). The plates were incubated overnight at 37°C. The next day individual (white) colonies were picked and expanded by culture as described before.

Preparation of RNA

For the preparation and all subsequent manipulations of RNA, all plasticware and solutions were treated or made in DEPC treated water. Total RNA and mRNA were prepared directly from cell pellets using Rneasy Total RNA System (Qiagen) and QuickPrep mRNA Purification Kit (Pharmacia Biotech), respectively according to the manufacturers instructions.

First strand DNA synthesis

mRNA to be amplified by PCR was reverse transcribed using the First-strand cDNA synthesis kit Pharmacia Biotech. The kit contains all components required for first strand cDNA synthesis, including a preassembled reaction mix containing Moloney Murine Leukaemia Virus (M-MuLV) reverse transcriptase, RNAGuard (an RNase inhibitor), RNase/DNase free BSA, and dNTPs in an aqueous buffer. An oligo-dT primer (Not I-d(T)₁₈ primer) supplied with the kit was used to prime cDNA synthesis; sequence as follows: 5'-d[AAC TGG AAG AAT TCG CGG CCG CAG GAA T₁₈]-3'. Oligo-dT primers bind to the polyA tail of mRNAs permitting synthesis of complementary strands by the reverse-transcriptase. Typically from 500ng to 1µg of mRNA was reverse transcribed in a single reaction, at 37°C, following manufacturers instructions and maintaining proportional reaction volumes.

Polymerase chain reaction (PCR)

PCR is a powerful technique that allows specific amplification of DNA sequences, facilitated by gene specific primers. It enables the amplification of unknown DNA sequences as long as the primers flanking the sequence are specific enough. In the present project PCR was used either for the modification of cloned DNA sequences, for example by deletion of unwanted sequences or inclusion of particular restriction sites to the ends of the molecules to facilitate subcloning, but also for the isolation of previously unknown sequences by using primers derived from homologous gene sequences of other animal species. A recent review on PCR, its applications and corresponding protocols can be found in Dieffenbach and Dveksler, 1995.

PRIMER DESIGN

For the design of PCR primers the following guidelines were observed. Primers were generally 18 to 28 nucleotides in length, with a G+C composition of 50 to 60% where possible. For a given primer pair, the annealing temperatures (T_m), were balanced and complementary regions between and within primers were avoided, minimising the production of primer-dimers. Also, target sequences in the gene with secondary structure (high G+C content) were not selected for priming sites.

MASTER MIXES

Master mixes are the reaction components (enzyme, buffer and dNTPs) excluding specific primers and DNA template. The relative proportion of components in a reaction mix is subject to optimisation in order to achieve a balance between specificity and quantity of amplified product. As a general rule decreasing the dNTP, primer and/or Magnesium content of a reaction mix increases the specificity of amplification accompanied, however by a decrease in product yield. All master mixes were set up in a departmental designated "PCR clean area" taking precautions to avoid PCR contamination such as the use of filter pipette tips. PCR was performed using either Taq polymerase (isolated from *Thermus aquaticus*) contained in the kits Gene Amp PCR Core (Perkin Elmer) or Pfu polymerase (from *Pyrococcus furiosus*) supplied by Stratagene. Care was taken to use the full contents of one kit at one meeting, avoiding freeze-thaw cycles. All the reagents were mixed at appropriate concentrations in an eppendorf tube, aliquoted in 500ul Perkin Elmer DNase free reaction tubes and stored at -20°C until used. The composition of individual reaction mixes as well as specific cycling conditions are detailed in each chapter.

Sequencing

Cycle sequencing reactions were carried out using IRD800-labeled primers (MWG-Biotech) and the ThermoSequenase Fluorescent Labeled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Life Science). Cycle sequencing is based on the dideoxynucleoside triphosphate chain termination method (Sanger, 1977) using a thermostable DNA polymerase that allows multiple rounds of high temperature DNA synthesis. Briefly, 400ng of plasmid DNA, 1pmol of IRD800 labelled primer and 2µl of reaction mix which contains 45mM each of dGTP, dATP, dTTP and dCTP, reaction buffer and thermostable DNA polymerase, were mixed in a 0.5ml reaction tube, to a total volume of 8µl. Cycling conditions were: 95°C for 5 min followed by 25 cycles of 95°C for 30 seconds, 50 to 60°C for 30 seconds and 72°C for 30 seconds. The annealing temperature was generally 2°C below the melting temperature of the primer used (details in individual chapters). Formamide loading buffer (4µl), supplied with the kit, was added and the reactions separated on denaturing gels, Sequagel XR ultra pure concentrate (National Diagnostics). Data recording was

performed on a Li-Cor model 4000 DNA sequencer (MWG-Biotech). Typically 500 to 700bp of good sequence was generated from one run.

DNA sequence data was analysed using the GCG package (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin).

RNA hybridisation analysis

NORTHERN BLOT TRANSFER OF RNA

15µg of total cellular RNA was lyophilised (VR-1 Hetovac, Heto), resuspended in 20µl of RNA loading buffer and denatured for 15 min at 65°C. Subsequently, 3 to 5µl of RNA loading buffer was added and the samples electrophoresed for 3 hours at 100V in 200ml of a 1% agarose gel containing 1 X MOPS and 2.2M formaldehyde. The gel running buffer contained 1X MOPS buffer and was continually recirculated. Following electrophoresis the gel was washed twice in ddH₂O for 20 min to remove formaldehyde, then equilibrated in 10 X SSC and transferred overnight onto Hybond-N (Amersham Life Science) in 10 X SSC. The membrane was removed and rinsed briefly in 2 X SSC and the RNA crosslinked to the membrane (Spectrolinker XL-1500 UV Crosslinker, Spectronics Corporation). All northern blots were probed with a rat gapdh probe to control for RNA loading and integrity. The GAPDH probe, kindly supplied by Dr E. Baxter (Department Veterinary Pathology, Glasgow), is a 750bp *Eco*RI fragment purified from the plasmid pGapdh.

PREPARATION OF RADIOLABELED DNA PROBES

PCR or restriction fragment DNA probes were gel-purified and radioactively labelled using a random primed DNA labeling kit (High Prime, Boehringer Mannheim), and $\alpha(^{32}\text{P})$ dCTP, specific activity 3000Ci/mmol (Amersham). Generally, 20-50ng of heat denatured DNA was radiolabeled using 50mCi (1.85Mbq) of $\alpha(^{32}\text{P})$ dCTP and 4µl of High Prime in a final volume of 20µl, following the manufacturer's instructions. Unincorporated nucleotides were removed by gel-filtration through Sephadex-G50 beads (Nick Columns, Pharmacia biotech) and labelled fragments were eluted in 400µl of TE buffer.

HYBRIDISATION AND FILM EXPOSURE

Standard high stringency conditions for the hybridisation of specific radiolabeled probes on nucleic acids immobilised on nylon membranes were as follows. Membranes pre-wetted in 2 X SSC, were rolled into Hybaid hybridisation bottles and pre-hybridised in 20ml of RNA pre-hybridisation buffer (see section 2.1.12) at 42°C for at least 2 hours in a Hybaid oven with continual rotation. Freshly boiled DNA probe (100µl) was added to the pre-hybridisation solution and the filters were hybridised overnight as above. After rinsing briefly with 2 X SSC, the membrane was washed for 20 min and with three changes of 0.1X SSC, 0.5% SDS at 60°C. Membranes were then sealed in polythene and exposed to X-ray film (Kodak) and following overnight exposure films were developed (Kodak X-omat processor ME-3).

Protein methods

ESTIMATION OF PROTEIN CONCENTRATIONS

In order to estimate protein yields a modified Bradford assay was used. This assay utilises the fact that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm following binding to protein. This shift in absorbance is linear over a relatively broad range of protein concentrations so allows accurate protein quantification within a sample.

A protein standard (bovine serum albumin or bovine gamma globulin) was diluted with dH₂O to concentrations ranging from 0.1 to 1.5 mg/ml to enable the production of a standard curve each time the assay was performed. Into individual wells of a flat-bottom 96 well plate was added 10µl of each test sample, 10µl of PBS (blank control) and 10µl of each dilution of the BSA standard curve. 200µl of Bradford dye reagent (Bio-rad) was added to each well and mixed thoroughly. The dye reagent was prepared by addition of 4 volumes of dH₂O to 1 volume of dye and filtration through a 0.4 µm syringe filter (Gelman Sciences). The plate was incubated at room temperature for 30 min to 1 hour, and the OD₅₉₅ subsequently measured against the reagent blank. The absorbance of the protein standards was used to construct a standard curve from which an approximate concentration of the unknown samples could be read.

SDS - POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEINS

The separation and analysis of proteins was facilitated by one dimensional denaturing discontinuous gel electrophoresis. Proteins were denatured by boiling in the presence of SDS and β -mercaptoethanol. The sample is then loaded onto a discontinuous gel consisting of a stacking buffer which concentrates the loaded protein sample into a narrow band and a separating gel which separates proteins on the basis of molecular size.

Minigels (8.0 x 7.3 cm) were produced and run using the Mini-PROTEAN II electrophoresis system (Biorad, Herts, UK) as recommended by the manufacturer. Glass plates were assembled with 0.75 mm spacers in a casting stand. The separating gel (12 to 15%) was poured to a depth of approximately 5 cm. Gel constituents and concentrations can be found in Sambrook et al, 1989 (page 18.52). This was overlaid with tris-saturated butanol and allowed to polymerise. The butanol was then poured off, the surface of the separating gel rinsed with dH₂O and the stacking gel poured. A 10 well comb was inserted and the gel allowed to polymerise. The gel was then transferred to the electrophoresis tank, both buffer tanks filled with running buffer, the comb removed and the wells flushed. Protein samples (typically 5 - 20 μ g of protein in 5 - 25 μ l) were prepared by addition of an appropriate volume of 6 x protein sample loading buffer, followed by heating to 100°C for five minutes. Samples were then loaded onto the gel using 0.2 mm flat ended gel loading tips. A protein molecular weight standard (5 -10 μ l) was loaded in one or both outer wells to allow estimation of the size of sample proteins.

Gels were electrophoresed at 140V for 60 to 80 minutes until the bromophenol blue dye reached the bottom of the separating gel. The gel was then removed from the glass plates, the stacking gel discarded, and the protein bands detected either by staining with Coomassie blue or by immunodetection.

GEL STAINING

Visualisation of protein bands with Coomassie blue involved staining for two hours in four to five gel volumes of protein fix-stain solution, followed by destaining for approximately 12 - 16 hours in destain solution. Gels were then removed and

preserved by drying for two to six hours, sandwiched between pre-wetted cellulose film, in a gel drying apparatus (Bio-Rad) for 2 hours at 80°C.

DETECTION OF PROTEINS BY IMMUNOBLOTTING

The detection of proteins by immunoblotting (western blotting) is a rapid and sensitive technique that exploits the inherent specificity of antigen recognition by antibodies. Proteins were transferred to PVDF membrane by electroblotting, following electrophoretic separation and detected using ECL reagents (Amersham Life Science). This detection system is based on the emission of light following the oxidation of luminol by horseradish peroxidase (HRP labelled antibodies), in the presence of chemical enhancers such as phenols. The light emitted can be detected by a short exposure to blue-light sensitive film (Hyperfilm ECL, Amersham).

Following SDS-PAGE, the gel was removed from the glass plates and rinsed in TBS. Hybond-ECL membrane was pre-wetted in 100% methanol for 15 seconds and then allowed to equilibrate with transfer buffer for 10 minutes prior to blotting. Proteins were transferred to the membrane using a semi-dry electroblotting system (Transblot SD - Biorad). The gel and membrane were sandwiched in close apposition between two sheets of extra thick filter paper (Biorad) pre-soaked in transfer buffer, and transferred at 10 V for 45 minutes.

The membrane was rinsed in TBS and non-specific binding sites blocked by immersing the membrane for 30 minutes in 10% low fat dried milk (Marvel - Premier Beverages, Stafford, UK) TBS-T (0.1% Tween in tris buffered saline) solution at room temperature on an orbital shaker. The membrane was rinsed briefly with TBS-T, washed once for 15 minutes then twice for five minutes, with shaking at room temperature. The membrane was then incubated with the primary antibody, at a pre-determined dilution (generally 1/10000) in 5% low fat dried milk, TBS-T, for 1 hour at room temperature, with shaking. The membrane was then washed as detailed above prior to incubating with the secondary antibody (HRP labelled), appropriately diluted (1/5000) in 5% low fat dried milk, TBS-T, for 45 min to 1 hour, at room temperature, with shaking.

The membrane was washed before detection by the ECL method, which was carried out in a darkroom, following manufacturers instructions. The membrane was placed, protein side up, in a film cassette and a sheet of autoradiography film (Hyperfilm-ECL) placed on top, in the dark. The cassette was closed and the film exposed for 15 to 30 seconds, before developing in an automated processor. A second sheet of film was then exposed, generally for 2 to 20 minutes, the time being estimated from the appearance of the first autoradiograph.

In order to confirm that the bands seen on Western blots were not due to non-specific binding of the secondary antibody, the blots were 'stripped' by soaking in 10 ml stripping buffer (6.25 mM Tris-HCl (pH 6.8), 2% SDS, 100 mM 2-ME) for 30 minutes, at 50°C, with agitation. The membrane was then reprobed, as above, however the primary antibody labelling step was omitted. Any non-specific binding of the secondary antibody was then detected by the ECL method, as outlined above.

CHAPTER 3

EXPRESSION AND BIOACTIVITY OF EQUINE INTERFERON GAMMA

3.1 Introduction

A comprehensive review on the structure and biology of IFN- γ can be found in chapter 1. The present chapter is focused on the practical aspects of the expression and assessment of biological activity of equine IFN- γ .

Since the cloning of human IFN- γ was attained (Gray *et al*, 1982), IFN- γ cDNAs from a number of species have been cloned, including: murine (Gray and Goeddel, 1983); bovine (Cerretti *et al*, 1986); porcine (Dijkmans *et al*, 1990); canine (Zucker *et al*, 1992) and feline (Argyle *et al*, 1995). Many of these groups went on to produce recombinant IFN- γ and to reproduce the results pioneered from murine and human studies in the assessment of its biological activity *in vitro* and *in vivo*. The main body of evidence related to the biological activity of natural and recombinant IFN- γ was gathered in the murine model. Nevertheless, the development of *in vitro* studies has facilitated the establishment of important parallels for the biological activity of IFN- γ in humans and other species with that observed in mice. IFN- γ was shown to stimulate an array of cellular responses *in vitro* that have been explored to assess the presence of biologically active IFN- γ in recombinant protein preparations and in clinical samples of a number of species (reviewed in De Maeyer and De Maeyer-Guignard, 1998). These activities are generally species specific and include: antiviral activity; anti-proliferative activity over tumour cells; induction of MHC class I and class II antigens; activation of macrophages for tumour cell and intracellular parasite killing; enhancement of NK cell activity; induction of B cells for antibody secretion.

The protocols used in this chapter are therefore adaptations of techniques developed for the study of IFN- γ in humans and mice, but successively applied in other animal models.

3.1.1 Equine Interferon Gamma

The cDNA for equine IFN- γ (eqIFN- γ) was originally cloned in the Department of Veterinary Pathology, Glasgow by Curran *et al* (1994). The cloning procedure entailed, in summary, the recovery of mRNA from mitogen stimulated peripheral blood lymphocytes and its amplification by RT-PCR using primers derived from conserved regions of the IFN- γ coding sequence of a number of species. The amplified cDNA was then cloned into a Bluescript plasmid and sequenced.

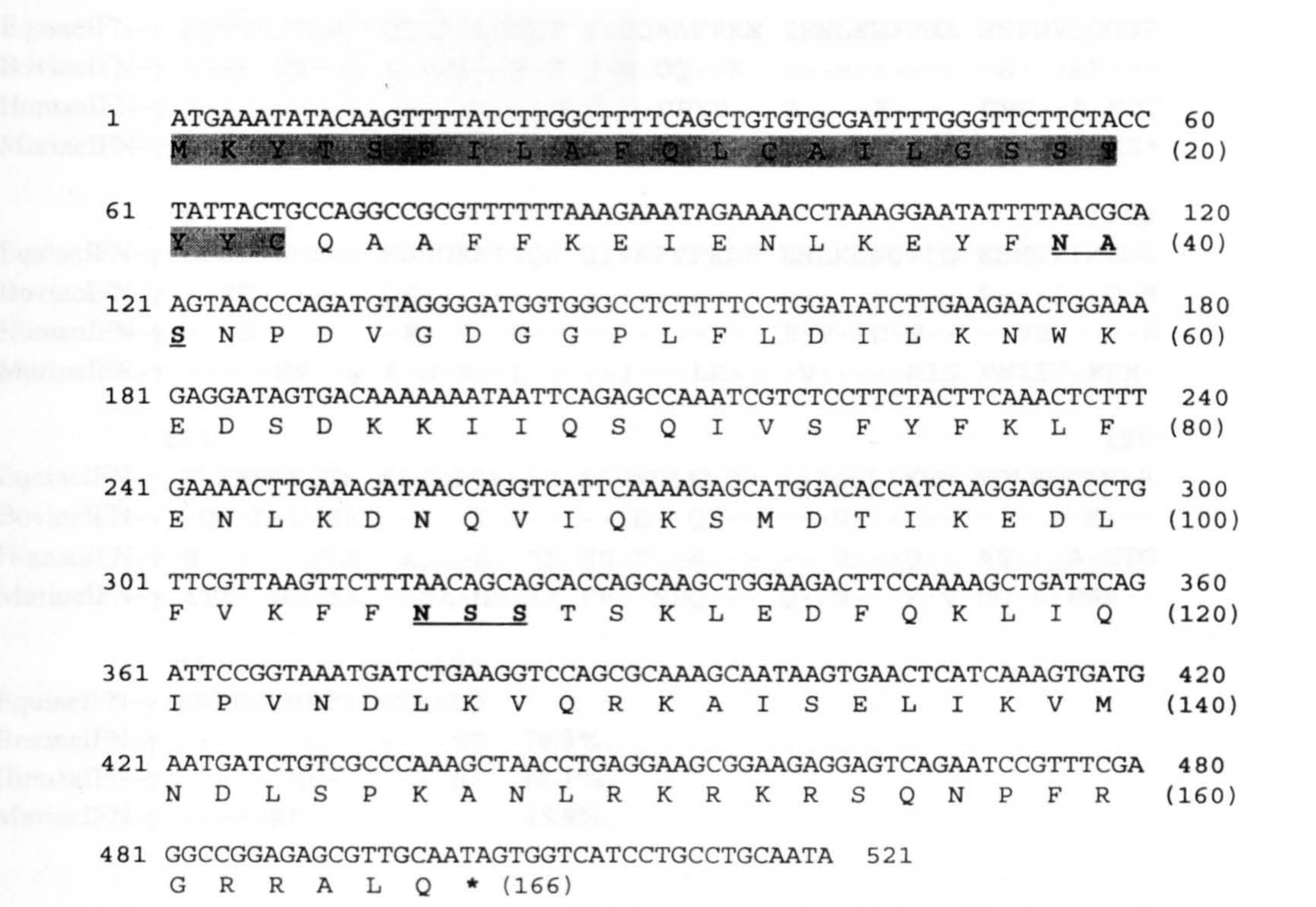


Figure 3.1 EqIFN- γ nucleotide sequence and corresponding predicted amino acid sequence. Signal peptide is shadowed and potential N-linked glycosylation sites underlined. The stop codon is marked by (*). Numbers correspond to nucleotide positions. Amino acid residues are numbered in brackets.

Analysis of the eqIFN- γ coding sequence revealed a 498 nucleotide long open reading frame with a homology of 66, 78 and 83% towards the murine, human and bovine

counterparts, respectively. At the amino acid level these homologies were of 45.5, 67.5 and 78%, respectively (figures 3.1 and 3.2). The predicted protein product is a 166 amino acid polypeptide featuring two potential N-linked glycosylation sites at residue positions 39 and 106. The sequence features a potential signal peptide sequence of 23 residues, by comparison to what was established in relation to human IFN- γ . Additionally the equine sequence 150RKRRKRSQ156 is similar to the murine RKRRKRSR, recently reported to be necessary and sufficient for nuclear translocation of IFN- γ (Subramaniam P. *et al*, 1999).

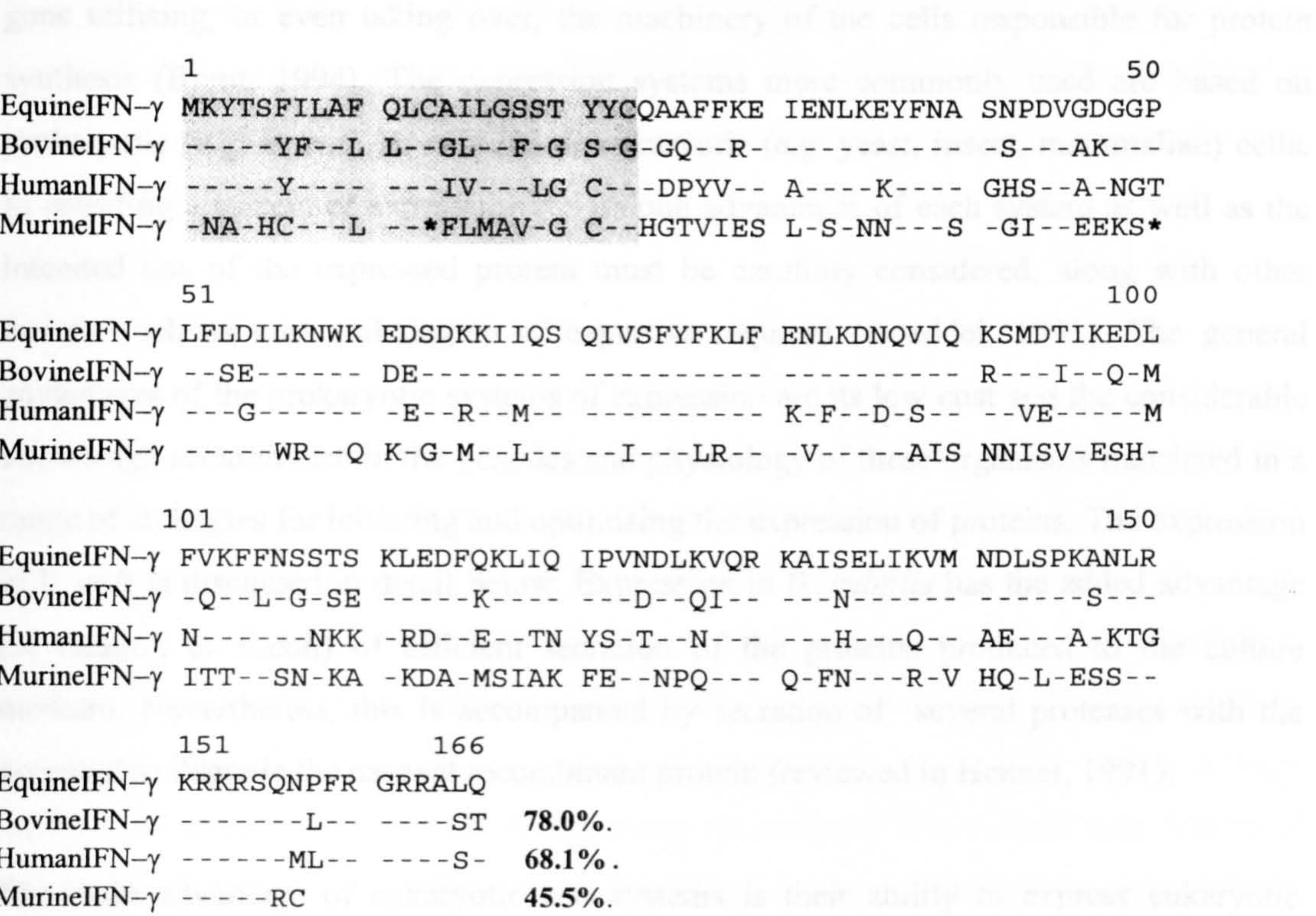


Figure 3.2 Comparison of the eqIFN- γ predicted amino acid sequence to that of other species. The percentage of homology to the equine protein is shown. Shaded box corresponds to the putative signal peptide sequence. (*) represents missing residues in the murine sequence. Numbers correspond to amino acid positions.

This conservation of features and the degree of homology suggest a similar role for eqIFN- γ to that observed in other mammalian species.

3.1.2 Expression of eqIFN- γ

3.1.2.1 Systems for heterologous gene expression

Recombinant proteins are proteins produced in heterologous gene expression systems through the introduction of the gene of interest carried by a vector (e.g. plasmid, virus) into living cells. The vector, containing an appropriate promoter, directs expression of the gene utilising, or even taking over, the machinery of the cells responsible for protein synthesis (Brent, 1994). The expression systems more commonly used are based on prokaryotic (e.g. *E. coli*, *B. subtilis*) or eukaryotic (e.g. yeast, insect, mammalian) cells. In selecting a system of expression the unique advantages of each system as well as the intended use of the expressed protein must be carefully considered, along with other factors such as cost and degree of expertise required (Goeddel, 1991). The general advantages of the prokaryotic systems of expression are its low cost and the considerable knowledge accumulated in the genetics and physiology of these organisms translated in a range of strategies for initiating and optimising the expression of proteins. The expression in *E. coli* is discussed in detail below. Expression in *B. subtilis* has the added advantage (in relation to *E. coli*) of efficient secretion of the proteins produced to the culture medium. Nevertheless, this is accompanied by secretion of several proteases with the potential to degrade the nascent recombinant protein (reviewed in Henner, 1991).

The main advantage of eukaryotic cell systems is their ability to express eukaryotic proteins in their native (or near-native) form due to efficient post-translational modification and secretion of the produced protein. The characteristics of the baculovirus and mammalian systems of expression are outlined below. Expression in yeast is based on the *Saccharomyces cerevisiae* strain, which can be propagated to very high cell densities and therefore produce large amounts of the protein of interest. However, high amounts of endo and exoproteinases gain access to the proteins in the yeast cytoplasm which results in extensive proteolysis of the recombinant proteins (reviewed in Erm, 1991).

3.1.2.2 Systems for expression of eqIFN- γ

Production of recombinant eqIFN- γ was accomplished using different systems of heterologous gene expression, namely, procaryotic, baculovirus and mammalian. The intrinsic properties of each individual expression system were explored to fulfil different end uses of the expressed protein. Thus, bacterial expression was used as a high yield source of IFN- γ of which crudely purified preparations could be easily obtained for inoculation and resulting production of antibodies. The baculovirus expression system was used for large scale production of biologically active IFN- γ and transient expression in mammalian cells was used as a verification system for the screening of constructs to be used in a DNA vaccination trial.

Expression in *Escherichia coli*

The expression of mammalian proteins in prokaryotes is a well established molecular biology tool, especially in the case of *Escherichia coli* (reviewed in Brent, 1994). Virtually any protein can be relatively fast and inexpensively produced in *E. coli*, as long as it is not too small, too large, too hydrophobic, too glycosylated or susceptible to proteolytic degradation. A number of expression systems based on *E. coli* have been developed and refined for the production of foreign proteins in high yields. A large amount of plasmid based systems of expression are currently available for introduction of foreign DNA into *E. coli*, efficiently orchestrating the expression of encoded proteins. Features shared by most of these systems include: a high copy number plasmid; a strong inducible promoter (e.g. lac, tac, trp); a potent translation initiation domain; a selectable marker (e.g. ampicillin resistance) that ensures maintenance of the vector and a multiple cloning site for insertion of the gene of interest (Goeddel, 1991).

A growing number of expression systems make use of fusion protein strategies (either N or C terminal) to increase the efficiency of expression. Reasons for the use of a fusion protein are: ensuring a good translation initiation; overcoming instability problems (especially of small peptides), increasing the solubility of the expressed protein and

reducing proteolytic degradation. A further reason for using fusion partners is often the ability to purify the expressed protein by affinity of the tags [e.g. histidine (His), trpE, glutathione S-transferase (GST)] to specific substances (e.g. metals, antibodies or substrates, respectively) (reviewed in Riggs, 1994)

The main disadvantage of the system resides in the fact that expressed proteins do not undergo post-translational processing including: glycosylation, phosphorylation, myristylation, signal peptide cleavage, etc. This can be overcome in some measure by the use of special host strains and intricate cloning strategies, but often these are time consuming and of limited success (Gold, 1991)

For the expression of eqIFN- γ in bacteria the glutathione S-transferase (GST) gene fusion system (Smith and Johnson, 1988) was chosen. This utilises a series of pGEX plasmids (Pharmacia, Biotech) featuring a tac promoter for isopropyl-1-thio- β -D-galactoside (IPTG) inducible high level expression and a lac I^q gene that enables its use in any *E. coli* host. Heterologous proteins are expressed as C-terminal fusions of *Schistosoma japonicum* GST. The translation of protein in pGEX-4T-1 begins at the ATG codon of the GST tag and fragments to be expressed are inserted in the polylinker downstream of GST. Therefore, care has to be taken in the preparation of inserts for cloning, as the reading frame has to be maintained.

Crude purification of fusion proteins is achieved by affinity chromatography and relies on the avidity of the GST tag to glutathione bound to a sepharose matrix. Recovery of GST tagged proteins is attained by the use of reduced glutathione under mild elution conditions, thus minimising procedural effects on the antigenicity and functional activity of eluted proteins. The plasmids also contain a thrombin recognition site (Gly-Pro-Arg) that allows cleavage of the protein of interest from the GST tag.

The host strain selected was BL21[DE3] (Stratagene) for increased protein expression and to minimize degradation of the expressed protein by *E. coli* proteases. BL21 strains are deficient in Lon and ompT proteases. Lon is a major ATP-dependent protease,

responsible for the degradation of naturally unstable or abnormal proteins (Gottesman, 1989). OmpT, localized in the outer membrane, is very active in *E. coli* extracts and cuts specifically at paired basic residues (Sugimura and Nishihara, 1988). The DE3 derivative contains the T7 RNA polymerase gene, controlled by the lacUV5 promoter for high level expression induced by IPTG.

Expression in Baculovirus

Expression in the baculovirus system entails the substitution of a non-essential gene of the virus by the gene of interest, followed by propagation of the recombinant virus in susceptible insect cells.

Baculoviruses that have been developed as expression vectors were originally isolated from the alfalfa caterpillar (*Autographa californica*) and belong to the *Baculoviridae* family of viruses that infect arthropods. Their large, double stranded, circular DNA genome is enclosed in a nucleocapsid, which is further packaged in a lipoprotein envelope to form the virus particle. Multiple nucleocapsids are occluded in a crystalline matrix (polyhedron), consisting largely of polyhedrin, hence the name (AcMNPV) *Autographa californica* multiple nuclear polyhedrosis viruses (King and Possee, 1992). In the natural virus life cycle, polyhedrin protects viral particles from proteolytic digestion as the host decomposes, although it is not required for production of infectious virus particles in cell culture. It is expressed in extremely large quantities at a very late stage of the replication cycle, making its promoter an ideal candidate to drive expression of foreign genes (Luckow and Summers, 1988).

The cell lines most commonly used for the replication of AcMNPV *in vitro* (Sf-9, Sf-21) were derived from pupal ovarian tissue of the “fall army worm” - *Spodoptera frugiperda* (Vaughn *et al*, 1977). When cells are exposed to infectious virus, two structurally distinct forms of virus are produced. The first, extracellular virus (ECV), responsible for the infection of neighbouring cells, begins to bud at 12 hours post-infection (p.i.) and features a glycoprotein (gp67) responsible for the attachment of virus to susceptible cells. The

second form, polyhedra-derived (occluded), lacks gp67 and appears within the nucleus of infected cells after 18 hours p.i., accumulating until released by cell lysis, up to 5 days p.i. The ECV is 1000 fold more infectious than the occluded form of virus (Volkman *et al*, 1986), whereas the occluded form produces larger amounts of polyhedrin, and therefore of foreign protein.

The advantages of the baculovirus system are that the genome can accommodate large amounts of foreign DNA without affecting normal replication and a high level of expression can be achieved using the strong polyhedrin promoter. Furthermore, the expression of very late gene promoters occurs in maturing infectious particles, which constitutes an advantage when expressing cytotoxic proteins, as they will not affect virus replication (Luckow and Summers, 1988). Also, the insect cells are able to perform most of the post-translational modifications required for biological activity of heterologous proteins. These include: glycosylation; phosphorylation; myristylation; palmitylation; proteolytic processing (including signal peptide cleavage) and secretion or cellular targeting. In the case of N-linked glycosylation, although being performed at the correct site (Asn-X-Ser/Thr) this is not done to the same extent as to that of mammalian cells. Recombinant glycoproteins expressed in insect cells are high in mannose but lack or have low levels of fucose, galactose and sialic acid residues (reviewed in King and Possee, 1992).

The system used is composed of a polyhedrin deficient AcMNPV (BAK-PAK6) and a transfer vector. The viral DNA, has been modified to include the *E. coli* β -galactosidase (lacZ) gene downstream of the polyhedrin promoter, in substitution of the polyhedrin gene. A unique Bsu361 enzyme recognition site was engineered within the lacZ gene (Possee *et al*, 1991) for the generation of linearized virus, following observations that this greatly increases the recombination efficiency (Kitts *et al*, 1990). The transfer vector (pAcCl29.1) consists of a bacterial plasmid containing the polyhedrin gene promoter and transcription termination elements flanked by AcMNPV sequences. It also features a polylinker sequence, downstream of the promoter, for insertion of foreign DNA (Livingstone and Jones, 1989). Co-transfection of insect cells with linearized infectious

viral DNA (AcMNPV-lacZ) and transfer vector containing the gene of interest, allows homologous recombination to occur, with consequent generation of recombinant virus. Recombinants are identified by blue/white selection in the presence of X-gal in a standard plaque assay. The blue plaques correspond to β -gal expressing parental virus, while white plaques correspond to recombinant virus in which the lacZ gene has been substituted by foreign DNA.

Expression in Mammalian cells

The broad advantages of using mammalian cells to express proteins from higher eukaryotes stem from the fact that the signals for synthesis, processing, and secretion of these proteins are usually correctly recognised. This authenticity of modifications reduces the risk of the expressed protein having altered biological activity, pharmacokinetics, or immunogenicity, which are factors of particular relevance when *in vivo* administration is planned. (Levinson, 1991; Brent, 1994).

There are two general methods of introducing genetic information to be expressed into mammalian cells. The first method is mediated by virus infection and has the advantage of efficiently targeting (near 100%) susceptible cells by exploiting the infectious capability of the virus. Nevertheless, a draw back to this strategy is the difficulty inherent in the engineering of the virus to express a foreign gene without compromising its replication and infection ability (Levinson, 1991). The second method is mediated by direct DNA transfection and utilises plasmid DNA as a vector of transfer. An advantage to this system is the commercial availability of a great variety of expression vectors constructed around known promoter (e.g. Rous Sarcoma Virus, Cytomegalovirus) and RNA processing signal sequences and containing polylinker cassettes for trouble free insertion of foreign cDNA. Its biggest disadvantage is the poor efficiency of DNA transfer, with only 5 to 50% of the cells acquiring and transiently expressing DNA. Although this may be circumvented by the laborious and time consuming procedure of isolating and characterising stably transfected cell lines (Kaufman, 1991).

The system used for mammalian expression of eqIFN- γ is based on the pCI-neo vector (Promega) for constitutive expression of genes in mammalian cells. The system can be used both for transient expression and for stable expression when transfected cells are selected with the neomycin analogue geneticin (G 418). The pCI-neo vector features: the human cytomegalovirus (CMV) immediate-early enhancer/promoter region for constitutive expression; the bacterial neomycin phosphotransferase gene allowing for selection of stably transfected cells; the SV40 origin of replication for transient high copy number episomal replication in cells expressing the SV40 large T antigen; a chimeric intron for increased expression levels, composed of the 5' splicing element from the β -globin intron and the 3'-splice site from an IgG intron; the SV40 late polyadenylation signal for efficient RNA processing and a multiple cloning site for insertion of foreign DNA.

The Cos7 cell line (Gluzman, 1981) was used for transfection. These cells are derived from African green monkey kidney cultures that were transformed with SV40 virus carrying a defective origin of replication and are therefore unable to produce viral particles. The cells produce large amounts of the viral protein, SV40 large tumour (T) antigen which directs in *trans*, high level amplification (10,000 to 100,000 copies per cell) of vectors containing the SV40 origin of replication. Consequently, plasmids containing a cDNA encoding a desired protein, under the control of an appropriate promoter, express large amounts of the protein in a short period of time. Typically protein production starts 24 hours post-transfection and lasts for up to a week, after which the cells either die or lose the plasmid (reviewed in Aruffo, 1994).

3.1.3 Biological activity of recombinant eqIFN- γ

3.1.3.1 Assays for cytokine detection

Existing methods for the *in vitro* detection and measurement of cytokines can be classified in two groups. The first group, immunoassays, although useful as a specific and sensitive way to quantitate cytokines, do not provide information on the bioactivity of the

detected cytokine as they also detect biologically inactive molecules. Immunoassays are based in the immunoreactivity of cytokines to specific antibodies (e.g. enzyme-linked immunosorbent assay-ELISA, radioimmunoassay- RIA) and can therefore be easily performed (Thorpe *et al*, 1992) . The second group, bioassays, are the only methods of cytokine detection that enable an estimation of the biological potency of the cytokines present in the test samples. Bioassays make use of cellular systems (cell lines or primary cultures) that require a certain cytokine for optimal growth (factor-dependent cell lines) or, more commonly, exploit different cellular functions dependent or enhanced by cytokine stimulation (Mire-Sluis *et al*, 1998). These functions include cell proliferation and the production of particular molecules that might be secreted (e.g. further cytokines and other intercellular mediators) or expressed in the surface of the cell (e.g. cytokine receptors, adhesion molecules, MHC). The suitability of a given cell line for assaying a particular cytokine depends on factors such as the ease of production and maintenance and sensitivity of response to the cytokine to be tested (Wadhwa and Thorpe, 1998; Mire-Sluis and Thorpe, 1998).

3.1.3.2 Biological assays for recombinant eqIFN- γ

Theoretically, every measurable function of IFN- γ can be considered for use as proof of biological activity. One has to attend, however, to more practical aspects like feasibility, availability of resources, time consumption and cost, when deciding which assay to use. The original and relatively simple assays based on the antiviral activity of IFN- γ in fibroblastic / epithelial cell lines have recently evolved to more complex and sensitive assays (e.g labelling of MHC upregulated molecules, intracellular staining of induced cytokines, measurement of nitric oxide production). This evolution is linked to continuous addition to the never ending list of intra and intercellular mediators and cell markers that can be up or downregulated by IFN- γ and to the development of powerful biological resources (e.g. monoclonal antibodies, transgenic mice).

The assays employed for investigation of biological activity of the eqIFN- γ preparations *in vitro* were chosen to reflect various aspects of IFN- γ activity.

EqIFN- γ cytopathic effect (CPE) reduction assay

This assay is based on the antiviral activity that IFN- γ shares with type I IFNs, albeit to a lesser extent. It relies on the cellular response to interferon and consequent inhibition of replication of an infecting virus, which can be measured in terms of reduction of cytopathic effect (CPE) in comparison to cells not treated with IFN, hence the name CPE reduction assay (reviewed in Meager, 1987).

The degree of protection conferred by the IFN preparation correlates with its potency and can therefore be used to titre individual preparations in terms of protective units. A unit is defined as the reciprocal of the dilution at which 50% protection against virus infection is obtained. The accuracy of the unit determination depends on the dilution increments and number of replicates. These are designated laboratory units and are extremely arbitrary, varying with virus stocks, cell lines, media, cell culture conditions and even operator. When uniformity of preparations is an absolute requirement, titrations should be calibrated to international reference standards provided by the World Health Organisation (reviewed in Lewis, 1987).

In deciding on the format of the assay, care has to be taken in the selection of a cell line sensitive to both the virus and responsive to IFN- γ and of a virus sensitive to the antiviral state of the cells. The virus chosen was the Vesicular Stomatitis Virus (VSV). Two cell lines were used: initially Equine Embryonic Kidney (EEK) and at a later stage Madin Darby Bovine Kidney (MDBK), both of epithelial-like phenotype.

VSV is a rhabdovirus that infects cattle, horses pigs and humans of which three serotypes have been isolated to date (VSV-New Jersey the most common, Indiana and Alagoas). In cattle VSV causes a disease clinically indistinguishable from Foot and Mouth disease with typical vesicles following scarification of the tongue and footpads. Occasional human infections with VSV have been reported, mostly from close contact with infected cattle or from laboratory exposures. In humans the disease is subclinical or mild and flulike (Dietzschold *et al*, 1996). The virus used in the CPE reduction assay is a

temperature sensitive mutant (VSVtsE2) of the New Jersey strain, containing two independent mutations in the NS gene (Rae and Elliott, 1986). The ideal growth temperature is 31°C and this strain is therefore unable to produce disease in livestock, or indeed to sustain a transmission cycle between animals.

SAFETY ASPECTS

In Scotland, the use of VSV is restricted to laboratories with category II status or superior, being additionally dependent on licence and report of periodical inspections by the Scottish Office, Agriculture and Fisheries Department. The departmental Code of Practice recommends: avoiding the use of sharps or blades; avoiding forming aerosols; before leaving the designated laboratory, all working surfaces are to be swabbed with 2% Virkon followed by 70% ethanol and all non-sterile material autoclaved immediately.

Proliferation assay for EqIFN- γ

This assay is based on the proliferation activity that IFN- γ exerts over T cell and B cell lines particularly when triggered by mitogens (Landolfo *et al*, 1988).

In the present assay, equine PBLs primed with IL-2 and concanavalin A (ConA) are stimulated with preparations of IFN- γ with or without basal amounts of ConA. The basal concentration of ConA is one that alone does not lead to proliferation. The measurement of proliferation relies in the incorporation of radioactive thymidine (H^3 -Thymidine) by the newly synthesised DNA in multiplying cells. The radioactivity retained by the cells is proportional to the level of proliferation and can be measured in a scintillation counter by the addition of a scintillation fluid to the cells.

MHC-II Upregulation by EqIFN- γ

This assay is based on the fact that IFN- γ can upregulate the expression of Major Histocompatibility Complex class II (MHC II) molecules in the surface of activated antigen presenting cells such as macrophages and dendritic cells (Wong *et al*, 1984).

While in most species MHC II epitopes are exclusively expressed by antigen presenting cells, in the case of the horse, circulating T cells also express significant amounts of equine MHC II (Crepaldi *et al*, 1986). Additionally, a large subset of circulating T lymphocytes (up to 95%) in the adult horse express MHC II, whereas in one day old foals expression of MHC II could not be detected (Lunn *et al*, 1993). This is comparable to the situation observed in humans in relation to the generation of CD45RO⁺ T cells that precedes T cell differentiation to a effector/memory phenotype (reviewed in Altin and Sloan, 1997). The constitutive expression of equine MHC II in T cells was therefore associated with the activation and/or development of memory of T cells in the horse (Lanzavecchia, 1990). Cells expressing MHC II are labelled with a monoclonal antibody against equine MHC class II and a secondary anti-mouse antibody conjugated with fluorescein. Quantitative and qualitative variations of MHC II expression by the cell population can then be assessed using a fluorescence-activated cell sorter (FACS).

Below is a description of the techniques used for the expression, detection and assay of biological activities *in vitro* of eqIFN- γ and the results obtained.

3.2 - Materials and Methods

3.2.1 Expression in *E. coli*

Cloning of eqIFN- γ into pGEX expression vectors

For the production of recombinant eqIFN- γ protein in *E. coli*, cDNA's encoding a full length protein and a truncated version lacking the signal peptide sequence were cloned (figure 3.1). The predicted mature version was prepared due to fears that the high content in hydrophobic residues of the leader sequence would reduce solubility of the expressed protein and therefore decrease its yield following purification.

PREPARATION OF EqIFN- γ CDNA INSERTS

Full length eqIFN- γ cDNA was recovered from pBlueScript (pBS) plasmid as follows. 10 μ g of pBS-eqIFN- γ was digested in a 50 μ l volume using 20 units of EcoRI and 5 μ l of EcoRI buffer at 37°C for four hours. The reaction was stopped by the addition of 6 μ l of 10X DNA loading buffer (mat&meth). The sample was loaded onto a 1.2% TAE agarose minigel, stained with ethidium bromide, and electrophoresed at 90 volts for 1 hour. Upon UV visualisation, a band of approximately 500bp in length, as determined by comparison to a DNA molecular weight standard (ϕ X174 RF DNA/Hae III fragments), was excised and the DNA extracted using Wizard PCR Prep (Promega) and eluted in 50 μ l of dH₂O (Figure 3.3- A). The truncated version of eqIFN- γ (IFNtr) was obtained by PCR amplification for which the following primers were designed:

Primer	Sequence	Tm
5' Primer	5'-CTG CCA GGC CGC GTT TTT TA-3'	62°C
3' Primer	5'-GGC AGG ATC ACC ACT ATT GC-3'	62°C

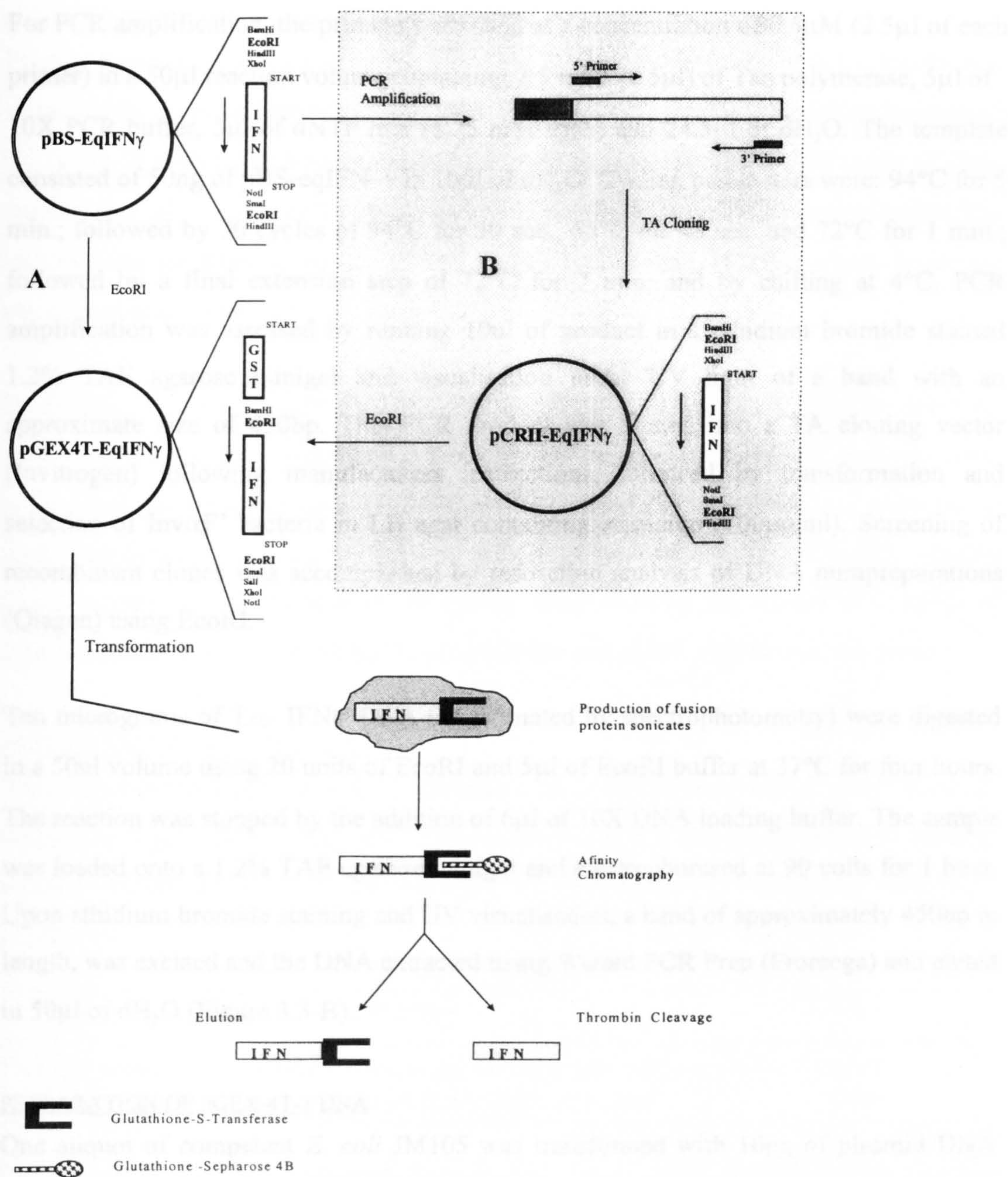


Figure 3.3 - Schematic showing cloning of eqIFN- γ in pGEX-4T-1 and major steps in the production and purification of recombinant eqIFN- γ in bacteria.

For PCR amplification, the primers were used at a concentration of 0.5 μ M (2.5 μ l of each primer) in a 50 μ l reaction volume containing 2.5 units (0.5 μ l) of Taq polymerase, 5 μ l of 10X PCR buffer, 5 μ l of dNTP mix (1.25 mM each) and 24.5 μ l of dH₂O. The template consisted of 50ng of pBS-eqIFN- γ in 10 μ l of dH₂O. Cycling parameters were: 94°C for 5 min.; followed by 30 cycles of 94°C for 30 sec., 55°C for 45 sec. and 72°C for 1 min.; followed by a final extension step of 72°C for 7 min. and by chilling at 4°C. PCR amplification was assessed by running 10 μ l of product in a ethidium bromide stained 1.2% TAE agarose minigel and visualisation under UV light of a band with an approximate size of 450bp. This PCR product was ligated into a TA cloning vector (Invitrogen) following manufacturers instructions, followed by transformation and selection of Inv α F' bacteria in LB agar containing ampicillin (100 μ g/ml). Screening of recombinant clones was accomplished by restriction analysis of DNA minipreparations (Qiagen) using EcoRI.

Ten micrograms of TA- IFNtr DNA (as estimated by spectrophotometry) were digested in a 50 μ l volume using 20 units of EcoRI and 5 μ l of EcoRI buffer at 37°C for four hours. The reaction was stopped by the addition of 6 μ l of 10X DNA loading buffer. The sample was loaded onto a 1.2% TAE agarose minigel and electrophoresed at 90 volts for 1 hour. Upon ethidium bromide staining and UV visualisation, a band of approximately 450bp in length, was excised and the DNA extracted using Wizard PCR Prep (Promega) and eluted in 50 μ l of dH₂O (Figure 3.3-B).

PREPARATION OF pGEX-4T-1 DNA

One aliquot of competent *E. coli* JM105 was transformed with 10ng of plasmid DNA and, following selection by ampicillin (50 μ g/ml) on LB agar plates, a single colony was grown in large scale for preparation of DNA by Qiagen plasmid mega kit. Ten micrograms were digested with EcoRI, and the linearised plasmid was gel purified as described above.

LIGATION OF VECTOR TO INSERTS AND TRANSFORMATION OF BACTERIA

Prior to ligation the DNA concentration of the linearised vector and both the inserts was estimated by spectrophotometry. To obtain a insert : vector molar ratio of approximately 5:1, 50ng of each insert was combined with 100ng of vector in a total volume of 20 μ l, containing 1 μ l (1 units) of T4 DNA ligase (Gibco, Brl) and 4 μ l of 5X ligase buffer. The reaction was incubated overnight at 15°C. A control ligation containing only linearised vector was also performed.

Competent *E. coli* JM105 were transformed with 10 μ l of each ligation reaction. Following overnight selection on LB agar with ampicillin (50 μ g/ml) colonies were picked and DNA minipreparations (Qiagen) were made and the DNA was submitted to restriction enzyme digestion followed by gel electrophoresis. The use of EcoRV enabled the screening of recombinants in the correct orientation by the release of a bands of approximately 1700 and 1750 bp for pGEX-IFN α and pGEX-eqIFN- γ respectively.

SEQUENCING OF RECOMBINANTS

Two clones containing each of the recombinant plasmids were sequenced using “Thermo-Sequenase Cycle Sequencing Kit” (Amersham) and the following IRD800 labelled primers (kindly supplied by Dr. S. Dunham, Department of Veterinary Pathology, Glasgow):

Primer	Sequence	Tm
5' pGEX primer	5'-GGG CTG GCA AGC CAC GTT TGG TG-3'	76°C
3' pGEX primer	5'-CCG GGA GCT GCA TGT GTC AGA GG-3'	76°C

Cycle sequencing was performed in a Perkin Elmer 9600 thermal-cycler. Cycling conditions were: 94°C for 5 min followed by 25 cycles of 95°C for 30 sec; 55°C for 30 sec. and extension at 72°C for 40 sec; followed by chilling at 4°C. Sequencing reactions were run in a DNA automated sequencer (Li-Cor 4000L) using gel running conditions, solutions and buffers specified in the accompanying protocols (mat&meth). One clone of each recombinant, showing 100% identity with published equine IFN- γ sequence (Curran *et al*, 1994), was taken through protein expression procedures.

Expression of eqIFN- γ fusion proteins

For this purpose, competent BL21[DE3] cells were transformed with recombinant plasmids, selected as detailed above and single colonies were picked for protein production. Cells were also transformed with parental plasmid for use as a control of the purification procedure.

SCREENING BY SMALL SCALE PROTEIN EXPRESSION

At this point the recombinant bacteria were screened for the production of detectable amounts of fusion proteins to investigate if the level of expression was sufficient to justify large scale purification procedures. Thus, 3ml cultures of the each construct were grown overnight in LB (with 100 μ g/ml of ampicillin and 1g/litre glucose) at 37°C with shaking. Cultures were diluted 1:10 into 5ml of the same medium and re-grown under the same conditions for 2.5 hours. The 5 ml cultures were divided in two fractions and to one of the fractions IPTG was added to a final concentration of 0.4mM. Both fractions of cells (induced and not induced) were incubated for another 2.5 hours. Cell extracts were prepared by pelleting 1.5ml of each fraction of the cultures and resuspending the pellet in 200 μ l of 1X Protein loading buffer (mat&meth). Samples were then boiled for 5 min. and spun in a microcentrifuge for 10 min. An 10 μ l aliquot of each sample was loaded in a sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) gel and analysed by Coomassie blue staining (mat&meth).

PREPARATION OF LARGE SCALE BACTERIAL SONICATES

A single colony of each recombinant and one colony containing empty plasmid were used to inoculate 20ml of 2YT medium containing 100 μ g/ml ampicillin and grown overnight at 37°C with vigorous shaking. The cultures were diluted 1:50 into a total of 400ml of pre-warmed 2YTA medium and grown with agitation at 30°C until the optical density reached approximately 1.0 at A₆₀₀. Cultures were induced with IPTG to a final concentration of 0.1mM and incubated for a further 4 hours.

The cultures were transferred to 500ml centrifuge bottles (Beckman) and centrifuged at 8000rpm for 10 min. in a JA-10 rotor to pellet the cells. The supernatants were discarded and the pellets drained and resuspended in 20ml of ice cold PBS containing the protease inhibitor AEBSF [4-2-(aminoethyl)-benzenesulfonyl fluoride hydrochloride] at 1mM. AEBSF is commercially available as Pefabloc SC (Boehringer Mannheim) and is a non-toxic water soluble alternative to the commonly used PMSF (Phenylmethylsulfonyl fluoride). Using a sonicator (XL 2020 - Heat Systems Inc.) equipped with an appropriate probe, the bacterial suspensions were sonicated on ice in three bursts of 20 sec. separated by 1 min. Care was taken to avoid frothing which is a known cause of protein denaturation. The degree of cell disruption was evaluated by darkening and decreasing of the suspension viscosity. To aid solubilisation of the fusion protein, 20% Triton X-100 was added to a final concentration of 1% and mixed gently at 4°C for 30 min. The suspension was transferred to polypropylene tubes and centrifuged at 18000 rpm for 10 min. in a Beckman JA-20 rotor. The supernatant was harvested to a clean tube and stored at -20°C until purification.

AFFINITY CHROMATOGRAPHY PURIFICATION OF FUSION PROTEINS

Prior to purification, 266 μ l of sepharose 4B slurry (Pharmacia Biotech) was applied to each disposable chromatography column and equilibrated with PBS. For the calculation of the amount of matrix needed the following figures were observed: Sepharose 4B is supplied as 75% slurry in 20% ethanol and in accordance with the manufacturers instructions 1.33ml of slurry is needed to produce one millilitre of bed volume; also 200 μ l of bed volume is needed for the purification of a sonicate derived from a 400ml culture. After equilibration a 50% slurry is obtained. The 20ml sonicates were added to the columns and allowed to flow through. The matrix is washed by the addition of 10 bed volumes of ice cold PBS and drainage. The wash step is repeated twice more for a total of 3 washes. At the end of the wash the O.D. at A280 of the flow through should be zero when compared to a blank of PBS. For elution of fusion proteins 0.5ml of elution buffer [10mM glutathione, 50mM Tris-HCl (pH 8.0)] was added to the matrix and incubated for 15 min. at room temperature before collection of eluate. The elution step was repeated twice and the three eluates were pooled and dialysed.

THROMBIN CLEAVAGE OF FUSION PROTEINS

Cleavage by thrombin releases the GST tag from the fusion protein. This is best done while fusion protein is still bound to the matrix, as cleavage releases the protein of interest for collection, whereas the GST tag remains attached to the matrix. To this effect the above protocol was followed until completion of the wash step. The column was sealed and 20 μ l of thrombin solution and 980 μ l of PBS was added to the washed matrix. The thrombin solution consists of thrombin at 1unit/ μ l in PBS and the manufacturer recommends the use of 50 units of thrombin per millilitre of bed volume. The matrix was then dislodged by tapping the column and incubated overnight with gentle rocking. Upon completion of incubation the column was centrifuged in a bench top centrifuge at 500rpm for a few seconds to sediment matrix. The bottom cap of the column was removed for collection of the eluate containing eqIFN- γ . The eluates were then dialysed.

DIALYSIS

Eluted proteins were dialysed against PBS to remove free glutathione and to equilibrate samples. Each eluate was loaded in a pre-soaked 3ml Slide-a-lyzer (Pierce) with a 10 kDa molecular weight cut off dialysis membrane. Dialysis was performed against PBS (1 litre per 1.5ml sample), for approximately 20 hours, at 4°C, with 3 changes of buffer. Dialysed samples were collected and stored at -70°C until further use.

IDENTIFICATION OF RECOMBINANT EqIFN- γ PREPARATIONS

Following crude purification and dialysis, recombinant proteins were analysed by Coomassie blue staining of SDS-PAGE gels. (mat&meth). Gels were assembled on a gel drier (Biorad) and dried at 80°C for 2 hours.

3.2.2 Expression in baculovirus

Production of Baculovirus-eqIFN- γ recombinants

A diagram outlining the major steps in the generation of recombinant Baculovirus-eqIFN- γ is shown in figure 3.4:

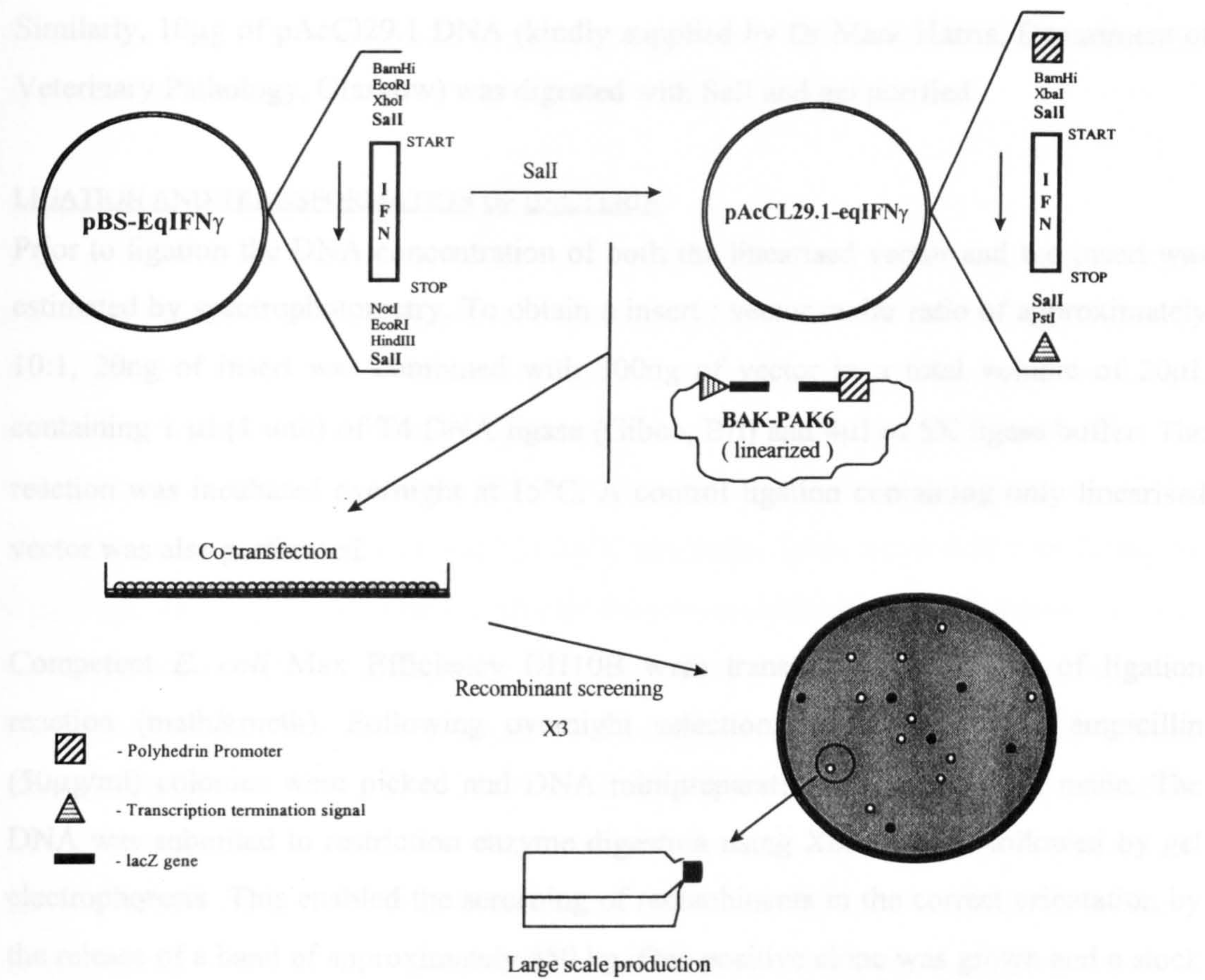


Figure 3.4 Schematic pinpointing major steps in the generation of recombinant Baculo-eqIFN- γ virus

CLONING eqIFN- γ IN pAcCL29.1

Full length eqIFN- γ cDNA was recovered from pBlueScript (pBS) plasmid as follows. 10 μ g of pBS-eqIFN- γ was digested in a 50 μ l volume using 20 units of Sall and 5 μ l of Sall buffer at 37°C for four hours. The reaction was stopped by the addition of 6 μ l of 10X DNA loading buffer (math&meth). The sample was loaded onto a 1.2% TAE agarose minigel, stained with ethidium bromide, and electrophoresed at 90 volts for 1 hour. Upon UV visualisation, a band of approximately 500bp in length, as compared to a DNA molecular weight standard (ϕ X174 RF DNA/Hae III fragments), was excised and

the DNA extracted using Wizard PCR Prep (Promega) and eluted in 50 μ l of dH₂O. Similarly, 10 μ g of pAcCl29.1 DNA (kindly supplied by Dr Mark Harris, Department of Veterinary Pathology, Glasgow) was digested with Sall and gel purified.

LIGATION AND TRANSFORMATION OF BACTERIA

Prior to ligation the DNA concentration of both the linearised vector and the insert was estimated by spectrophotometry. To obtain a insert : vector molar ratio of approximately 10:1, 20ng of insert was combined with 300ng of vector in a total volume of 20 μ l, containing 1 μ l (1 unit) of T4 DNA ligase (Gibco, Brl) and 4 μ l of 5X ligase buffer. The reaction was incubated overnight at 15°C. A control ligation containing only linearised vector was also performed.

Competent *E. coli* Max Efficiency DH10B were transformed with 10 μ l of ligation reaction (math&meth). Following overnight selection on LB agar with ampicillin (50 μ g/ml) colonies were picked and DNA minipreparations (Qiagen) were made. The DNA was submitted to restriction enzyme digestion using XbaI/Bsu36I followed by gel electrophoresis. This enabled the screening of recombinants in the correct orientation by the release of a band of approximately 450 bp. One positive clone was grown and a stock of pAcCl29.1-eqIFN- γ was prepared using "Plasmid Mega Kit" (Qiagen). Resulting DNA was analysed by ethidium bromide stained agarose gel and quantified by spectrophotometry.

PREPARATION OF VIRAL DNA

One microgram of AcMNPV DNA (provided by Dr Mark Harris) was digested in a 50 μ l volume using 10 units (1 μ l) of Bsu36I and 5 μ l of buffer III, at 37°C for 2 hours, followed by inactivation of the enzyme by heating at 65°C for 10min. Completion of digestion was verified by loading 5 μ l of the reaction in a 1.2% TAE agarose minigel, stained with ethidium bromide, and comparing it with uncut DNA. The remaining of the digested DNA was made up to 200 μ l with dH₂O and extracted with an equal volume phenol / chloroform / isoamyl alcohol [25:24:1(v/v)], precipitated in 10% 3M Sodium acetate (v/v) and 2.5 volumes of 100% ethanol. The DNA was pelleted by centrifugation

in a microfuge at 13000 rpm, washed with ice cold 70% ethanol (v/v) and freeze-dried. Resulting pellet was resuspended in 50 μ l of dH₂O and DNA concentration assessed by spectrophotometry.

CO-TRANSFECTION OF INSECT CELLS

Prior to transfection, SF-9 cells (kindly provided by Dr. Mark Harris) were grown and maintained (mat&meth) in log phase, characterised by doubling times of approximately 48 hours and viability over 90% between passages. Co-transfections were performed in triplicate. For each transfection a 35mm tissue culture dish was seeded with 10⁶ SF-9 cells in 2ml of complete medium [TC100 (Gibco, Brl), 10% Foetal bovine serum (FBS), 10 μ g/ml gentamycin] and incubated at 28°C overnight. In an eppendorf tube 200ng of viral DNA and 1 μ g of recombinant transfer vector were mixed in a total volume of 20 μ l. Lipofectin (Gibco, Brl) was diluted 2:1 (lipofectin : dH₂O) and 20 μ l combined with the DNA mixture and incubated for 20min. at room temperature. Meanwhile the cells were gently washed twice with 2ml of TC100 medium (without FBS or antibiotics) and left in 1ml of this medium. The liposome-DNA mixture was then added to the cells and after 5 hours of incubation at 28°C, 1ml of complete medium was applied and the cells incubated overnight. The medium was removed and substituted by 2ml of fresh complete medium and incubation was resumed until 48 hours post-transfection.

HARVEST AND PLAQUE PURIFICATION OF RECOMBINANT VIRUS

The cells were resuspended by vigorous pipetting and the suspension transferred to sterile eppendorf tubes and centrifuged in a microfuge at 13000 rpm for 5min. The supernatant containing a mixture of parental and recombinant virus was then harvested and filtered through a 0.45 μ m filter for plaque purification. The virus suspension obtained was added both neat and at a 10⁻¹ dilution in a volume of 100 μ l to 35mm tissue culture dishes seeded with 10⁶ Sf-9 cells and incubated for 1 hour at room temperature. Meanwhile sufficient 3% low melting point Seaplaque agarose (Flowgen) in dH₂O was melted and equilibrated at 45°C. The inoculum was removed and discarded and 2ml of 3% agarose mixed 1:1 with complete TC100 medium (at room temperature) was added to the cells and allowed to set. The solid agarose overlay was covered with 1.5ml of complete medium and the

plates incubated at 27°C for 3 to 4 days. A viable cell stain was prepared containing 0.1% neutral red (w/v) and X-gal (0.4mg/ml) in dH₂O. The stain was added to the overlaid cells (0.5ml per dish) and incubated for 1 hour at 27°C. The supernatant was removed and the dishes were inverted and left to stand overnight in the dark and at room temperature. A total of nine white plaques (three per co-transfection) were picked using sterile plastic Pasteur pipettes and resuspended into bijoux containing 2ml of complete medium. The bijoux were vortexed and stored at 4°C until further use. The resuspended virus passed through two more rounds of plaque purification. Three blue plaques (of parental virus) were also picked to serve as negative control in downstream procedures.

Screening for recombinant Baculo-eqIFN- γ virus

After each of the three rounds of plaque purification the resuspended plaques were screened for the presence of eqIFN- γ by PCR. A negative control consisting of a parental virus plaque suspension was used. Viral templates to be used in PCR amplification were prepared simply by boiling plaque suspension aliquots for 10min, centrifugation in a microfuge at 13000 rpm, and transferring supernatants to fresh tubes.

Primer	Sequence	Tm
5' Primer	5'- GCG TCC ACC ATG AAT TAT AC-3'	58°C
3' Primer	5'- GGA TAC TGT ATT GCA GGC AG-3'	60°C

The primers above were used at a concentration of 0.5 μ M (2.5 μ l of each primer) in a 50 μ l reaction volume containing 2.5 units (0.5 μ l) of Taq polymerase, 5 μ l of 10X PCR buffer, 5 μ l of dNTP mix (1.25 mM each) and 24.5 μ l of dH₂O. The template consisted of 1 μ l of boiled viral supernatants in 9 μ l of dH₂O. Cycling parameters were: 94°C for 5 min.; followed by 30 cycles of 94°C for 30 sec., 55°C for 45 sec. And 72°C for 1 min.; followed by a final extension step of 72°C for 7 min. and by chilling at 4°C. PCR amplification was assessed by running 10ul of product in a ethidium bromide stained

1.2% TAE agarose minigel and visualisation under UV light of a band with an approximate size of 500bp.

Propagation and titration of Baculo-eqIFN- γ virus

Two recombinants (1.3.1 and 3.5.1) were selected for propagation and “large scale” production of recombinant eqIFN- γ . To this effect, 100 μ l of each selected plaque suspension (from the third round of purification) was used to infect a 75cm² tissue culture flask seeded with 5x10⁶ SF-9 cells. After 1 hour of incubation at room temperature the cells were overlayed with 15ml of complete medium and incubated for 7 days at 27°C. The medium was harvested, freed of cell debris by centrifugation filtered through a 0.22 μ m filter and stored at 4°C. Titration was performed using the standard plaque assay described above. Dilutions of the virus were made (10⁻⁴, 10⁻⁵, 10⁻⁶ and 10⁻⁷) and plated in duplicate. To determine the virus titre the monolayers were stained and the plaques were counted in the dilution that enabled clear distinction of the plaques (dishes containing from 5 to 30 plaques). The titre is given in plaque forming units per millilitre (pfu/ml) and is calculated by the average number of plaques between the two dishes of the same virus dilution, multiplied by the dilution factor (10⁵, 10⁶, etc.) and by 10 (as only 0.1ml of virus dilution was applied to each dish). Propagation of baculovirus constructs was accomplished by infecting SF9 cells with a multiplicity of 0.1 to 0.2 pfu/cell. After one hour incubation and harvesting of the inoculum, cells were replenished with fresh medium and incubated at 27°C for 6 to 7 days. After three rounds of infection, the titres obtained for individual baculovirus constructs were: 3x10⁷ pfu/ml for Baculo.eqIFN- γ (1.3.1); 4.8x10⁷ pfu/ml for Baculo.eqIFN- γ (3.5.1); and 1.3x10⁷ pfu/ml for Baculovirus wild type.

Large scale production of eqIFN- γ in baculovirus

For production of baculovirus derived eqIFN- γ , healthy SF21 cells were infected as before with a multiplicity of 10 p.f.u / cell. Cells were incubated at 27°C for 3 and 4 days.

Incubation times were based on optimisation of expression of feline IFN- γ in baculovirus (Dr D. Argyle personal communication). The medium was then harvested by centrifugation, dialysed against PBS (1 litre per ml of supernatant, changed four times), using a 30ml 10kDa molecular weight cut off slide-a-lyser (Pierce), filtered through a 0.22 μ m filter, aliquoted and stored at -70C.

3.2.3 Expression in mammalian cells

Cloning eqIFN- γ into pCI-neo

Full length eqIFN- γ cDNA was recovered from pBlueScript (pBS) plasmid as follows (see figure 3.5). 10 μ g of pBS-EqIFN- γ was added to 20 units of XhoI and 20 units of NotI in a 100 μ l volume and digested at 37°C for four hours. The reaction was stopped by the addition of 12 μ l of 10X DNA loading buffer (mat&meth).

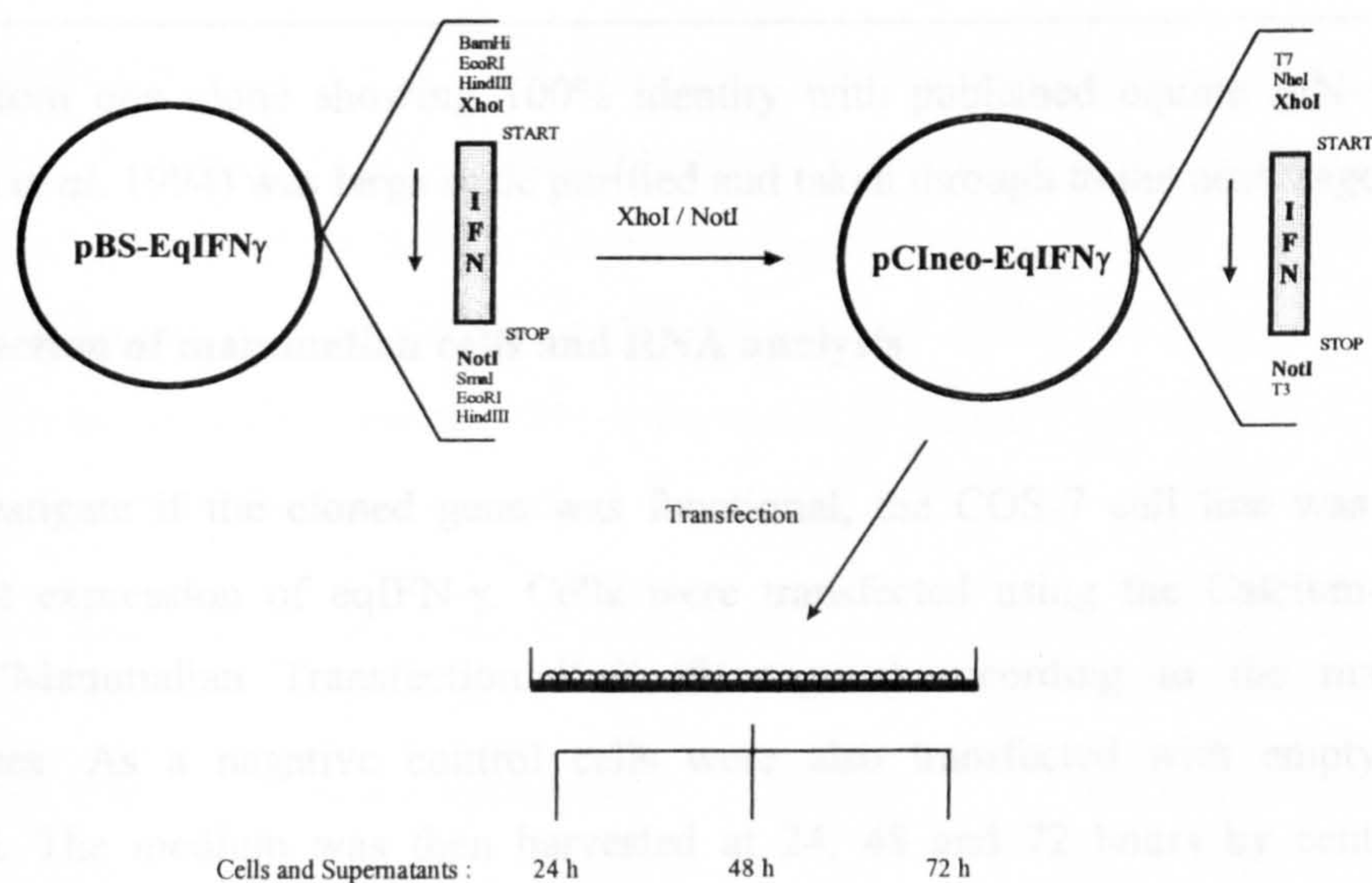


Figure 3.5 Schematic depicting the expression of eqIFN- γ in mammalian cells.

The sample was loaded onto a 1.2% TAE agarose minigel, stained with ethidium bromide, and electrophoresed at 90 volts for 1 hour. Upon UV visualisation, a band of approximately 500bp in length, as compared to a DNA molecular weight standard (ϕ X174 RF DNA/Hae III fragments), was excised and the DNA extracted using Wizard PCR Prep (Promega) and eluted in 50 μ l of dH₂O. Similarly, 10 μ g of pCI-neo DNA (Promega) was digested with XhoI/NotI and gel purified. Ligation of the fragments was then performed (as described before).

The *E. coli* strain Max Efficiency DH10B was transformed and recombinant clones were screened by restriction analysis of miniprep DNA, using XhoI/NotI enzymes followed by agarose gel electrophoresis, producing a band of approximately 500 bp. Two clones containing the correct size insert were sequenced by automated sequencing (mat&meth) using the following primers:

Primer	Sequence	Tm	Target
T 3	5'- CAT TAA CCC TCA CTA AAG GG -3'	58°C	Vector
T 7	5'- TTA ATA CGA CTC ACT ATA GG -3'	54°C	Vector

DNA from one clone showing 100% identity with published equine IFN- γ sequence (Curran *et al*, 1994) was large scale purified and taken through to the next stage.

Transfection of mammalian cells and RNA analysis

To investigate if the cloned gene was functional, the COS-7 cell line was used for transient expression of eqIFN- γ . Cells were transfected using the Calcium-phosphate based "Mammalian Transfection Kit" (Stratagene) according to the manufacturer guidelines. As a negative control cells were also transfected with empty pCI-Neo plasmid. The medium was then harvested at 24, 48 and 72 hours by centrifugation, filtered through a 0.22 μ m filter, dialysed against PBS (as described before) using a 30ml 10kDa molecular weight cut off slide-a-lyser (Pierce), aliquoted and stored at -70°C.

Detection of eqIFN- γ expression

Transfected cells were collected by trypsinisation and centrifugation at 24, 48 and 72 hour time points. Total RNA was extracted from cells using “Rneasy Midi Kit” (Qiagen) and contaminating DNA was removed by incubating the samples with 1 unit of DNase I (Gibco BRL) per μg of RNA for 20 min at 37°C followed by enzyme inactivation at 65°C for 5 min. and by phenol/chloroform extraction of the resulting RNA. The purified RNA was quantified by spectrophotometry and analysed by northern blot (mat&meth) using a gene specific radiolabelled probe. The probe, consisting of a fragment eqIFN- γ , generated by EcoRI digestion of Bluescript-eqIFN- γ plasmid DNA, was labelled with [α - ^{32}P]dCTP (Amersham) making use of the “Oligolabelling Kit” (Pharmacia). A probe for the house-keeping enzyme GAPDH (mat&meth) was used for quality control of the RNA preparations.

3.2.4 Immunological detection of eqIFN- γ

The detection of eqIFN- γ derived from the various heterologous expression systems, confirming the identity of the expressed products, was accomplished by means of specific antibodies against eqIFN- γ . In an attempt to explore the possibility of benefiting from commercially available antibodies for the detection of eqIFN- γ , a panel of monoclonal antibodies to human and murine IFN- γ expressed in bacteria was used in western blots but no cross-reactivity was observed.

Antibody tested	Antibody type	Supplier	Catalogue No.
anti-human IFN- γ	mouse monoclonal	Serotec	MCA1533B
anti-mouse IFN- γ	rat monoclonal	Serotec	MCA 1394
anti-human IFN- γ	mouse monoclonal	R&D	MAB 285
anti-human IFN- γ	mouse monoclonal	Chemicon	MAB408

The rationale of testing monoclonal antibodies rather than polyclonal serum was the possibility monoclonals brought of developing a capture ELISA suitable for quantitation of recombinant and natural equine IFN- γ preparations.

3.2.4.1 Antibodies to eqIFN- γ peptides

Antibodies specific to eqIFN- γ in the form of polyclonal rabbit sera were obtained using two strategies. The first involved the immunisation of rabbits with synthetic peptides and the second made use of GST-eqIFN- γ fusion protein for immunisation.

Design and Synthesis of eqIFN- γ peptides

To increase the probability of a peptide being immunogenic, a few simple guidelines are to be followed: peptides are ideally 10 to 20 residues in length, hydrophilic and/or flexible amino acids are more likely to be exposed in the surface of molecules therefore forming epitopes; the amino and carboxy termini are also generally exposed (reviewed in Harlow and Lane, 1988). To facilitate the design of peptides the nucleotide sequence of equine IFN- γ cDNA (Curran *et al*, 1994) was analysed using GCG software. The predicted amino acid sequence was analysed using the programs “Peptidestructure” and “Plotstructure” to display graphically (figure 3.6) the antigenic index as predicted by the Jameson and Wolf index (Wolf *et al*, 1987). The calculation of the antigenic index takes into consideration various predicted parameters, such as: N-linked glycosylation; hydrophilicity (according to Kyte and Doolittle); surface probability (Emini); chain flexibility (Karplus-Schulz); secondary structure (according to Chou-Fasman and Garnier-Osguthorpe-Robson).

Two regions of the molecule, with 20 amino acids in length and scoring a high antigenic index, were chosen to serve as immunogens:

Peptide 35 NH₂- AFFKEIENLKEYFNASNPDV- COOH (residues 26 to 45)

Peptide 36 NH₂- DLSPKANLRKRKRSQNPFR- COOH (residues 142 to 161)

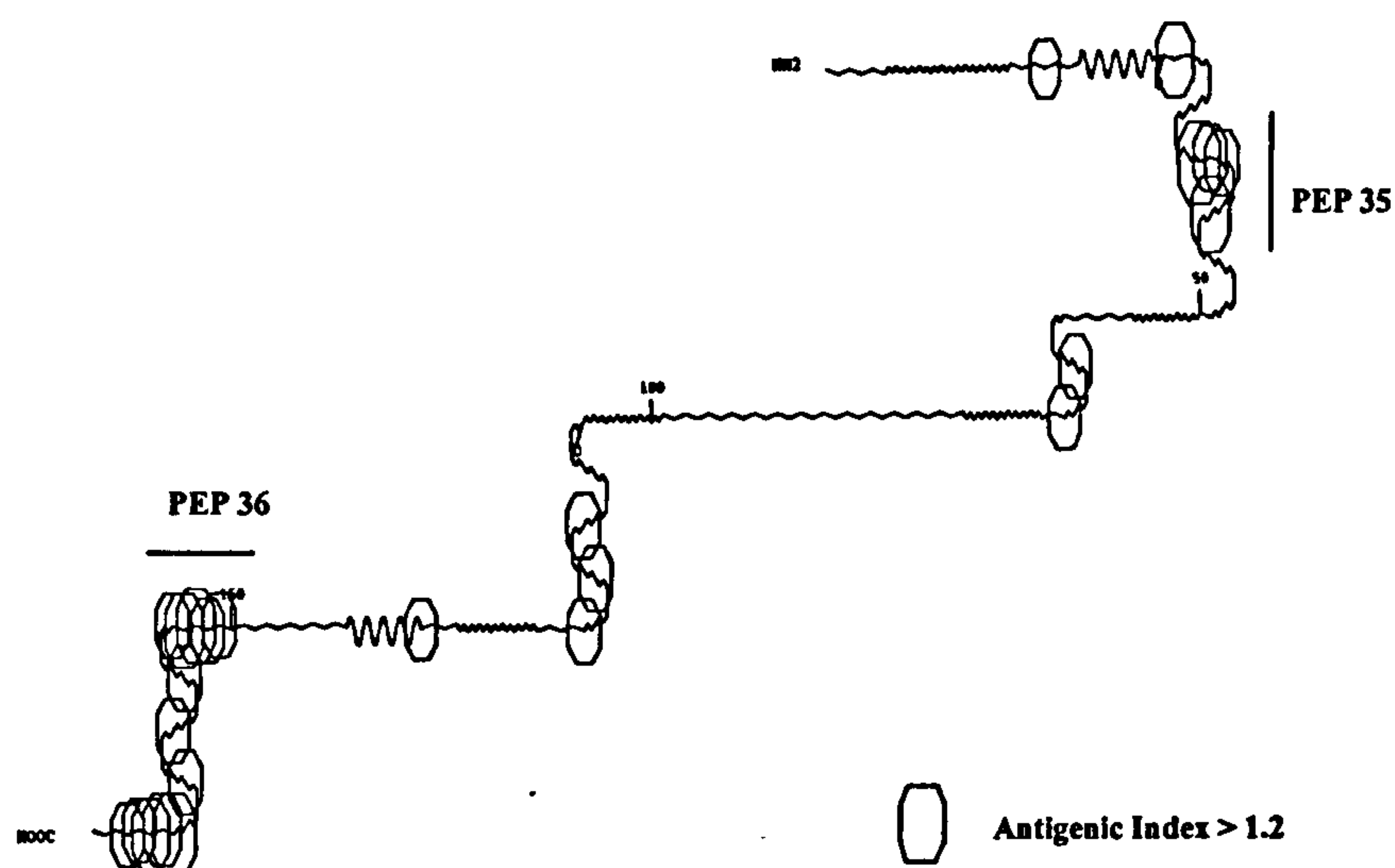


Figure 3.6 Analysis of eqIFN- γ predicted amino acid sequence using GCG software, showing predicted antigenic structure of the molecule (Wolf *et al*, 1987).

Both peptides correspond to regions of the human IFN- γ molecule thought to be involved in receptor binding, and are for that reason flexible structures, constituting therefore, potentially good immunogens (reviewed in 3.1.1).

1		50
MKYTSFILAF	QLCAILGSST	YYCQAAFFKE IENLKEYFNA SNPDVGDGGP
51		100
LFLDILKNWK	EDSDKKIIQS	QIVSFYFKLF ENLKDNQVIQ KSMDTIKEDL
101		150
FVKFFNSSTS	KLEDFQKLIQ	IPVNDLKVQR KAISELIKVM NDLSPKANLR
151	166	
KRKRSQNPFR	GRRALQ	

Figure 3.7 Amino acid sequence of eqIFN- γ showing peptide sequences selected for use as immunogens.

Peptides were synthesised using a multiple antigen peptide (MAP) system (Tam, 1988) consisting of a small inner core of branched lysine residues, to which multiple copies of the peptide antigen can be attached. The immunogenicity of peptides synthesised in this fashion is greatly increased due to the mobility conferred by the branching conformation of MAP. This system, when compared with the linear conjugation of peptides to carrier proteins (such as bovine serum albumin), has the additional advantages of not altering the folding of the peptides and because the carrier is internal it does not constitute an immunogen per se, therefore not contributing significantly to the total antibody response (Tam and Shao, 1993). Synthesis was performed in a 432A Peptide Synthesiser (Applied Biosystems) operated by Mr Tom Dunsford (Department of Veterinary Pathology, Glasgow). Peptide 35 and 36 were synthesised as multiple antigen peptides in a 4 branched lysine synthesis column using the Fast-moc procedure as detailed in the manufacturer instructions. Both peptides, obtained as freeze-dried preparations, were reconstituted in dH₂O to 1mg/ml, aliquoted and stored at -70°C

Preparation of immunogen and immunisation of rabbits

Immunogens were prepared by the combination of peptides with Freund's adjuvant (FA) that consists of non-metabolisable oils. The addition of the aqueous peptide solution produces a water in oil emulsion which prevents dispersion of the immunogen and generates a strong and durable non-specific immune response. For primary immunisations the peptides were combined with complete Freund's adjuvant (CFA) prepared by the addition of inactivated *Mycobacterium tuberculosis* vaccine (Bacillus Calmette-Guerin- BCG). Booster immunisations consisted of peptide combined with incomplete Freund's adjuvant (IFA) to prevent adverse side effects associated with repetitive inoculation of CFA, such as ulcerative and granulomatous lesions (reviewed in Harlow and Lane, 1988). To prepare CFA, 0.1ml of BCG vaccine (Evans Medical Ltd.) was added to 0.9ml of aqueous peptide solution. This mix was then combined with 2ml of non-ulcerative FA (NUFA - Guildhay Ltd.) to produce a water in oil emulsion. The procedure for preparation of IFA simply excludes the addition of BCG.

Four adult New Zealand white rabbits (two per peptide), were selected and prior to immunisation 15ml of blood, from each rabbit, was collected from the marginal ear vein into sterile glass universals. The blood was left at room temperature for a few hours and then left to clot overnight at 4°C. Serum was harvested and transferred to clean universals and residual red blood cells pelleted by centrifugation at 3000 rpm for 10 min. in a bench top centrifuge. The serum was then aliquoted (1ml aliquots) and stored at -20°C. The pre-immune serum derived from this blood was to be used as a negative serum control. The immunisation using a standard protocol is outlined below. Each inoculation consisted of a total volume of 1ml, administered subcutaneously, divided between four sites:

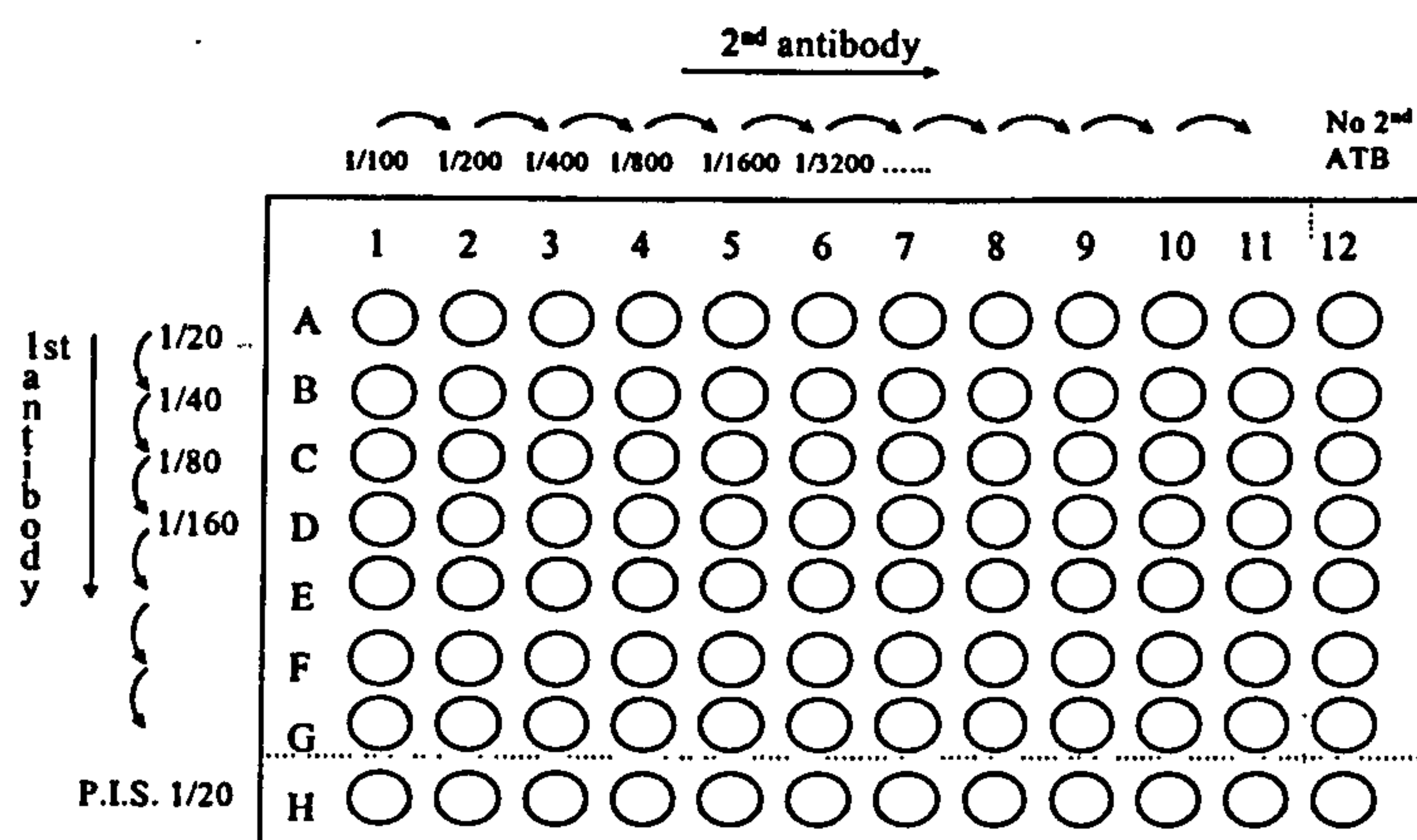
Rabbit	V579	V693	V566	V606
Peptide	35	35	36	36
1 st Immunisation (CFA)	100µg	100µg	100µg	100µg
4 WEEKS				
Booster (IFA)	100µg	100µg	100µg	100µg
14 DAYS				
Serum Collection	V579	V693	V566	V606

Ten to fifteen millilitres of blood was collected 14 days post-immunisation, and respective serum prepared as before. All immunisations and bleedings were performed by Home Office Licence holder Dr David Argyle (Department of Veterinary Pathology, Glasgow).

Indirect antibody ELISA

An indirect antibody enzyme linked immunosorbent assay (ELISA) was performed to verify the specificity and sensitivity of the sera in the detection of the respective peptides and to assess cross reactivity between sera and peptides. For this purpose an ELISA starter kit (Pierce & Warriner) which consists of coating and washing buffers; goat anti-rabbit IgG (H+L) antibody, conjugated with horseradish peroxidase and the enzyme

substrate [2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS)] was used. Initially each antiserum was assayed in a bi-dimensional titration procedure where both the primary and secondary antibodies were serially diluted in checker board fashion (see diagram below). To this effect peptides 35 or 36 were diluted to 5 μ g/ml in coating buffer (carbonate-bicarbonate buffer - BupHTM), 100 μ l was added to each well of a 96 well ELISA plate and the plates were incubated at room temperature for 1 hour. After incubation the plates were washed by addition of 100 μ l per well of wash buffer (modified Dulbecco's phosphate buffered saline with tween 20 and bovine serum albumin). The wash was repeated twice more for a total of three washes.



The plates were then blocked, for 30 min at room temperature, with 100ml per well of 1% BSA in wash buffer. To titrate the primary antibody, 100 μ l of wash buffer was added to each well of rows B to G and 190 μ l to row A. Then 10 μ l of serum to be tested was added to each well of row A (to give a 1/20 dilution). The antibody was serially diluted 1/2 (by pipetting 100 μ l) from row A to G. To row H was added a 1/20 dilution of pre-immune serum (P.I.S.) in wash buffer, as a negative control. Plates were incubated for 1 hour at room temperature and washed three times as before. The secondary antibody is titrated in the same fashion, but this time starting in column 1 and with a 1/100 dilution. Column 12 is left without secondary antibody as a negative control. Plates were incubated for 30 min. at room temperature, washed three times and 100 μ l of the enzyme substrate (ABTS) was added to each well. After 30 min of incubation plates were read at an absorbance of 405nm using an ELISA plate reader model EL312 (Bio-tek Instruments Inc.).

A further ELISA assay was devised to investigate if each sera had unique specificity for the peptide used as immunogen or if, on the other hand, they were cross reactive. This time, though, information collected in the previous assay led to optimisation of the dilutions of primary and secondary antibodies used (see diagram below). Thus, rows A to D were coated with peptide 36, rows E to G with peptide 35 and row H was left uncoated. Columns 1 to 9 contained the primary antibody serially diluted twofold from 1/200 through to 1/51200. To column 10 no primary antibody was added and columns 11 and 12 contained a 1/200 and a 1/800 dilution of pre-immune serum, respectively. Rows A and E; B and F; C and G, contained secondary antibody in decreasing concentrations (1/2500; 1/5000; 1/10000, respectively). Row D was negative control for the secondary antibody. Incubation times and washes were as before.

Coat.		2 nd ATB									No 1st ATB		P.I serum		
		1/200	1/400	1/800	1/1600	1/3200							1/200	1/800	
Pep 36	1/2500														A
	1/5000														B
	1/10000														C
	No 2nd ATB														D
Pep 35	1/2500														E
	1/5000														F
	1/10000														G
No Pep	1/2500														H
		1	2	3	4	5	6	7	8	9	10	11	12		

Detection of GST-eqIFN- γ by anti-peptide sera

A series of ELISA assays were performed to ascertain if anti-peptide antibodies would recognise mature eqIFN- γ . The assay was similar to the one described previously. In summary, each well of a 96 well plate was coated with 100 μ l of GST-eqIFN- γ at 10 μ g/ml (as assessed by Bradford). Two sera were tested per plate and were twofold serially diluted (1/200 through to 1/51200). Secondary antibody was used at different

concentrations (1/2500, 1/5000, 1/10000) as before. Sufficient wells were allocated for pre-immune antibody control and absence of secondary antibody.

3.2.4.2 Antibodies to GST-eqIFN- γ

Crudely purified GST-eqIFN- γ fusion protein (as described above, 3.2.1.1) was used to immunise rabbits and consequently raise polyclonal serum. The option of using the tagged protein (as opposed to the cleaved eqIFN- γ molecule) was justified by the reported highly immunogenic character of the GST tag (Campbell *et al*, 1995). Two adult New Zealand white rabbits (82 and 95) were selected for immunisation. The protocol for preparation of the immunogen, as well as the dose and route of administration were the same as used for the production of anti-eqIFN-peptide serum using 100 μ g of GST-eqIFN- γ per inoculation. Significantly the immunisation procedure for rabbit 82 consisted of a primary immunisation of GST-eqIFN combined with CFA, followed by three boosts (combined with IFA) at six weeks intervals. In the case of rabbit 95 only one boost was made, with thrombin cleaved eqIFN- γ . Bleeds were made two weeks after each immunisation. This work was executed in collaboration with Dr Jay Patel at Intervet UK (The Elms, Cambridge). On receipt, sera were labelled 82-A, 82-B, 82-C and 95-A. Sodium Azide, to a final concentration of 0.01% (w/v), was added to the sera, followed by aliquoting in 500 μ l aliquots and freezing at -70°C.

3.2.4.3 Detection of expressed products

For the immunological detection of eqIFN- γ produced in different expression systems, typically 10 μ l of each sample to be analysed was combined with 2 μ l of 10X protein loading buffer and ran on SDS-PAGE (mat&meth). Following transfer to a PVDF membrane and blocking, the polyclonal serum to GSTeqIFN- γ was used as primary antibody at a dilution of 1/10000. The secondary antibody was an anti-rabbit IgG peroxidase conjugate (Sigma A-6154) diluted to 1/5000. ECL reagents (Amersham) were used to develop blots. The full protocol for western blot is described in mat&meth.

Potency of signal after ECL detection, proportional to the quantity of eqIFN- γ , was the main selection criterion for samples to be tested in *in vitro* bioactivity experiments. Additionally, within samples with equivalent amounts of recombinant protein, were selected those derived from shorter time point infections or transfections, in order to minimise degradation and loss of activity of secreted recombinant IFN- γ exposed to temperatures over 25°C. Also, as detailed before, samples were dialysed against PBS (pH 7.0), as pH values higher than 9.0 and lower than 5.0 lead to rapid loss of IFN- γ activity (Farrar and Schreiber, 1993). Particularly for baculovirus derived eqIFN- γ , the choice of samples was also influenced by findings obtained in the case of feline interferon gamma in this department (Dr D. Argyle personal communication).

Samples taken through assessment of biological activities were:

Baculovirus.eqIFN- γ (3.5.1)	72 hours infection supernatant	BacIFN72	TEST SAMPLES
Baculovirus.eqIFN- γ (3.5.1)	96 hours infection supernatant	BacIFN96	
PciNeo.eqIFN- γ *	24 hours transfection supernatant	PciIFN24	
PciNeo.eqIFN- γ *	48 hours transfection supernatant	PciIFN48	
Baculovirus wild type	72 hours infection supernatant	Bacwt	NEG.
Pci-neo*	48 hours transfection supernatant	Pcineo	CONTROLS

(*) Mammalian expression constructs were assayed at one instance only, in the cytopathic effect reduction assay.

3.2.5 Bioactivity in vitro of EqIFN- γ

3.2.5.1 Cytopathic effect reduction assay

The assay is loosely based in that described by Familletti *et al* (1981).

PREPARATORY PROCEDURES

A stock of VSV, originally adapted to feline embryonic fibroblasts (FEA) was kindly provided by Dr. David Argyle (Department of Veterinary Pathology). The virus was adapted to EEK and MDBK cell lines by consecutive passages of virus at a multiplicity of infection of 0.2 TCID₅₀/cell, until a high titre was obtained, as assessed by TCID₅₀. Cell growth experiments were made and the number of cells per well necessary for the production of a monolayer in 36 h was 2×10^4 for EEK and 1.5×10^4 MDBK.

VIRUS TITRATION

TCID₅₀ was performed by adding the above amount of cells per well of a 96 well plate and incubate for 20 h at 37°C and 5% CO₂. Logarithmic dilutions of the virus were made in complete cell medium (EMEM, 10% FBS, 2mM glutamine, 1X Penicillin/Streptomycin for EEK and MEM, 5% FBS, 2mM glutamine, 1X Non essential amino acids, 1X Penicillin/Streptomycin for MDBK) and incubation at 31°C for 72 h. Plates were scored by inverted microscopy at 10X magnification. The virus had a final titre of 1.5×10^6 TCID₅₀/ml in EEK and 1×10^6 TCID₅₀/ml in MDBK, as calculated by the formula:

$$\text{Log TCID}_{50} = D - x (S - 0.5)$$

where: D = log of the lowest dilution showing 100% CPE

x = difference between successive dilutions

S = sum of proportional mortalities (sum of the percentual score of mortality in successive dilutions from 100% to 0% CPE)

The time necessary for development of 100% CPE in the dilution corresponding to 100 TCID₅₀ was also noted (48 h).

ASSAY CONDITIONS

Twofold dilutions of the samples (BacIFN72/96 and PciIFN24/48) or of negative control samples (Bacwt and Pcineo) were performed in sufficient 96 well plates. Each sample was analysed in a different plate and wells were left without interferon to serve as cell and virus controls. Each well was then seeded with 2x10⁴ (EEK) or 1.5x10⁴ (MBDK) cells. Cells were incubated at 37°C for 20 hours. After incubation the supernatants containing interferon were removed and cells were gently washed twice with PBS, and fresh medium added to the cells. VSV virus was diluted and 100µl of each dilution was added to the plates as follows: 100TCID₅₀ were added in fourfold to wells corresponding to each interferon dilution; in the same fashion 10TCID₅₀ were added to threefold wells; a back titration of the virus was also performed in each plate; some wells were left free of virus to serve as cell controls (figure 3.8).

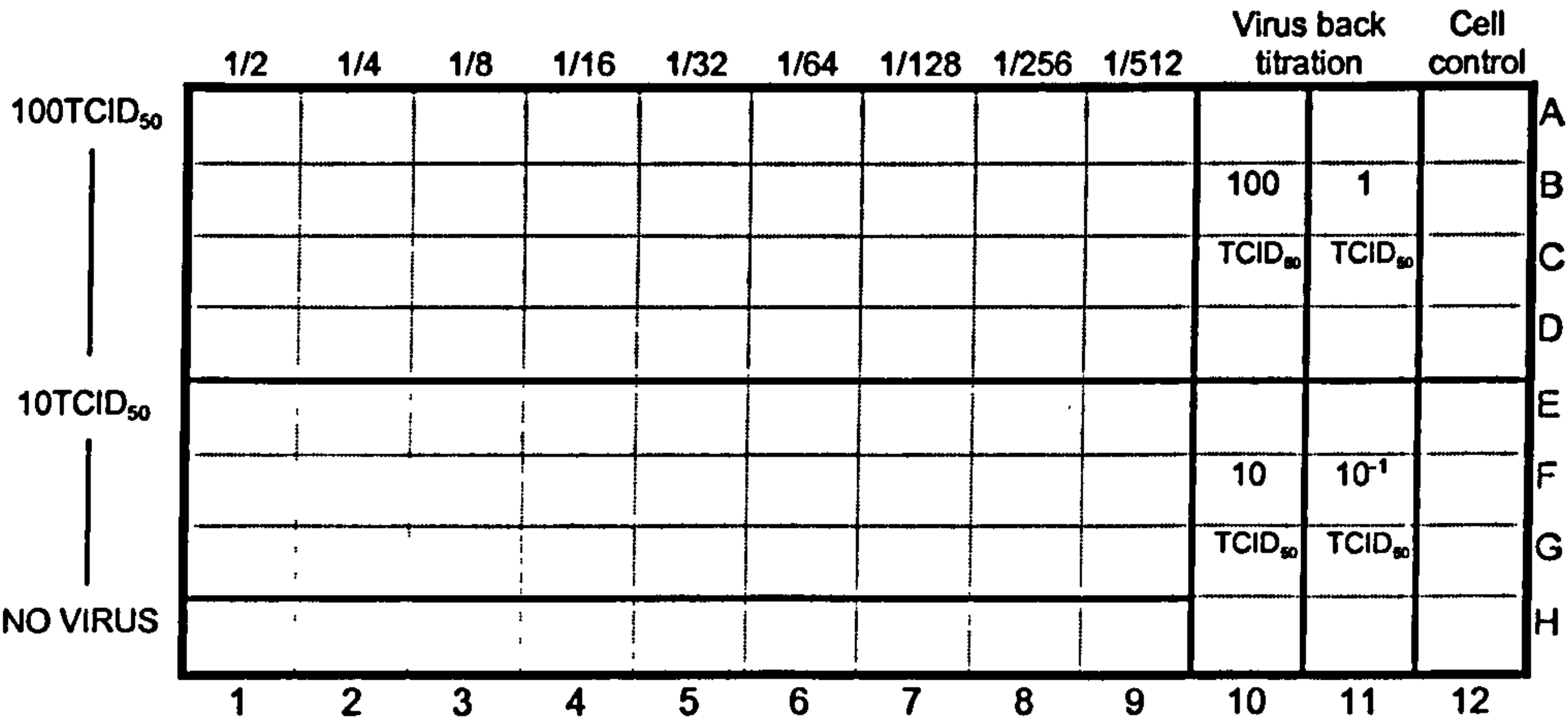


Figure 3.8 Cytopathic effect reduction assay setup.

Plates were incubated at 31°C until 100% CPE was observed in the virus control wells (48 h). The medium was removed from the wells and the cells stained / fixed by adding 50µl of 0.5% crystal violet in 70% methanol to each well and incubating 5 min at room

temperature. The excess stain was decanted and the plates rinsed thoroughly with tap water and air-dried at room temperature. Interferon laboratory titre of the samples was calculated as the reciprocal of the dilution of interferon that protected 50% of the wells from the CPE of the virus.

3.2.5.2 Proliferation assay for Equine IFN- γ

PREPARATION OF EQUINE PERIPHERAL BLOOD LYMPHOCYTES (PBLs)

Whole horse blood (SAPU Lanarks, UK) supplied 1:1 in Alsever's solution, was diluted with an equal volume of ice cold PBS (mat&meth) and layered onto a Ficoll gradient (Ficoll-Paque Plus, Pharmacia Biotech) in 50ml Falcon tubes and centrifuged at 1500g at 4°C in excess of 30 min. Cells in the interface were carefully aspirated, washed in an equal volume of PBS (by centrifugation at 1000g) and resuspended in 1ml of PBS per 10ml of original volume of blood. An equal volume of freshly prepared red blood cell lysis buffer (mat&meth) equilibrated at 37°C was added to the cell suspension and incubated with rocking movement at 37°C for under 4 min. The cells were then recovered by centrifugation as before and washed twice in complete lymphocyte medium (RPMI 1640 with 2% FCS, 100 IU/ml of penicillin/streptomycin, 10mM Hepes, 2mM Glutamine and 5×10^{-5} M β -mercaptoethanol). Cells were then counted, resuspended in freezing medium (90% FCS, 10% dimethyl-sulfoxide) to 1×10^7 cells/ml aliquoted in cryo-tubes (1ml aliquots) and frozen using a programmable cell freezer (Planer Kryol10) and a standard protocol for lymphocytes. On completion of the freezing cycle, cells were transferred to liquid nitrogen and stored until needed.

ASSAY CONDITIONS

The following protocol was modified from one used for the detection of murine IL-12 bioactivity in phytohaemagglutinin stimulated lymphoblasts (Schoenhaut *et al*, 1992). PBLs retrieved from liquid nitrogen were quickly thawed at 37°C, diluted in complete lymphocyte medium and incubated for 2 hours at 37°C and 5% CO₂. Cells were then recovered by centrifugation, counted and cultured at a density of 1×10^6 cells/ml in complete lymphocyte medium containing rhIL-2 and 5 μ g/ml of Concanavalin A (ConA)

(Sigma) and incubated at 37°C for 48 hours. Recombinant human IL-2 (rhIL-2) obtained as a supernatant of stably transfected LTK⁻ cells (T. Hattori *et al*), kindly supplied by Dr. Margaret Hosie (Department of Veterinary Pathology, Glasgow) was used for proliferation purposes at a 1/50 dilution (as suggested by Dr. Hosie).

The cells were harvested, washed twice in PBS, counted and resuspended at 1×10^6 cells/ml. Cells were then plated in sufficient 96 well plates at 50 μ l/well. Serial twofold dilutions of eqIFN- γ preparations BacIFN72 and BacIFN96 or negative control (Bacwt) were made in complete medium and 50 μ l added to each well in triplicate, in conjugation or not with 0.3 μ g/ml of ConA. This concentration of ConA was previously observed not to cause PBL proliferation.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1/2			1/2			1/2			5 μ g/ml		
B	1/4			1/4			1/4	Parental		2.5 μ g/ml		
C	1/8			1/8	eqIFN- γ		1/8	Vector		1.25 μ g/ml		
D	1/16	eqIFN- γ		1/16	+		1/16			0.62 μ g/ml	ConA	
E	1/32			1/32	ConA		1/2	Parental		0.31 μ g/ml		
F	1/64			1/64	(0.3 μ g/ml)		1/4	Vector		0.16 μ g/ml		
G	1/128			1/128			1/8	+ ConA		Cells		
H	1/256			1/256			1/16	(0.3 μ g/ml)		No cells		

Figure 3.9 Equine PBL proliferation assay setup.

A ConA standard curve, consisting of twofold dilutions varying from 5 μ g/ml to 0.16 μ g/ml added to dedicated wells of each plate in 50 μ l volumes, was also performed to serve as an intra-assay positive control. Additional negative controls consisting of wells with cells or medium only were also made. Plates were incubated for 48 hours at 37°C.

0.5 μ Ci [H^3] methyl-thymidine (Amersham) in 50 μ l of complete medium was added to each well and the cells were pulsed by incubation for 18 hours at 37°C. The cells were

then harvested into filters and washed four times, using a cell harvester (Packard Filter-mate 196) and oven dried at 60°C for 1 hour. 25 μ l of Microscint-O (Packard) scintillation fluid was added to each well. The amount of ³H-thymidine incorporated was assessed using a scintillation counter (Packard model A9904V).

3.2.5.3 MHC-II Upregulation by EqIFN- γ

For the purpose of the assay, an enriched population of T lymphocytes was stimulated with eqIFN- γ preparations and appropriate negative controls. The cells were then stained and analysed by flow cytometry for the presence of MHC II. The assay was based on the one described for recombinant canine IFN- γ (Zucker *et al*, 1993).

PREPARATION OF T CELLS

T cells were enriched by negative selection using a nylon wool column. For the preparation of columns, ready to use nylon wool (Polysciences) was teased apart to remove clumps using sterile technique. The wool was then packed into a syringe barrel (1.2g for a 10 ml syringe or 2.4g for a 20ml syringe), sealed in a autoclave bag and autoclaved (120°C for a minimum of 20 minutes).

Enough aliquots of PBL's (see above) stored in liquid nitrogen were quickly thawed at 37°C, diluted in complete medium (RPMI 1640, 2% FBS, 100 μ g/ml penicillin/streptomycin, 2mM glutamine, 5x10⁻⁵M B-mercaptoethanol) and incubated for 2 hours at 37°C and 5% CO₂. Cells were then centrifuged as before, resuspended in an appropriate volume of complete medium and counted as before and a single cell suspension at 1x10⁸ cells/ml in complete medium was prepared. The column was attached to a stand in a tissue culture hood and on the column outlet was placed a 3 way stopcock (BDH, Merck) and a 23-gauge needle. A minimum of 3 volumes of complete medium was run through the column removing any air bubbles and the column was equilibrated for 1 hour at 37°C.

The column was then flushed with additional complete medium and the cell suspension loaded (10ml column for up to 1.5x10⁸ cells and 20ml column for up to 3.0x10⁸ cells) and

the cells allowed to drain into the column. A further 1 to 3ml of complete medium was added and allowed to drain into the column after which the stopcock was closed and the cells (in the column) incubated for 1 hour at 37°C. The stopcock was opened and slowly (rate =1 drop/3secs) the cells were eluted in a minimum of 2 volumes of complete medium. The cells were collected in a 50ml Falcon tube, spun, washed once with complete medium and counted and resuspended at 2×10^6 cells/ml.

ASSAY CONDITIONS

A volume of 250 μ l of cells (5×10^5 cells) was dispensed in each well of a 48 well tissue culture plate. Serial logarithmic dilutions of the BacIFN72 preparation and appropriate control Bacwt were made in complete medium and a 250 μ l volume of the dilutions was added to consecutive wells and the plates incubated at 37°C with 5%CO₂ for 24 hours. Some wells were inoculated with complete medium only, as a cell control. In a separate experiment the same dilutions of BacIFN72 and negative controls were co-incubated with rhIL-2 at a dilution of 1/50 as described previously.

ANTIBODIES

Cells were stained by indirect staining, using as primary antibody mouse IgG1 anti-equine MHC II monoclonal antibody (Kydd *et al*, 1991) commercially available with the designation CVS 20 (Serotec) and as secondary antibody the F(ab')₂ fragment of sheep anti-mouse IgG (whole molecule) conjugated to fluorescein isothiocyanate (FITC) (Sigma). Primary antibodies often bind, through the Fc portion, to cells expressing FcR, resulting in an overestimation of the percentage of positive epitopes in the labelled cells. To investigate if this overestimation was significant, an isotype control, termed "irrelevant antibody", with no epitope specificity but with same isotype and subclass as the primary antibody (and therefore containing the same Fc determinant) was used. The irrelevant antibody, used in substitution of the primary antibody, as a negative control, was a mouse IgG1 anti-FIV p24 mAb (kindly supplied by Dr A. Muller, Dept. of Vet. Pathology, Glasgow). Also as negative controls, cells were stained in the absence of either primary or secondary antibody.

To determine the relative amounts of lymphocyte subsets in the cell population and therefore the purity of the T cells, the following antibodies were used: mouse IgG1 anti-equine CD5 mAb (Serotec) for staining of T cells; mouse IgG1 anti-equine CD4 mAb (Serotec) for T helper cells and mouse IgG2b anti-equine B cell mAb (Serotec) for B lymphocytes. A pre-assay was performed to verify if the cells could be stained with the selected antibodies and to make a rough titration of the antibodies to optimise the concentration of mAb to be used, minimising waste. For this purpose unstimulated cells were stained (as described below) with different amounts of primary mAb (30, 15, and 7.5 μ l) and combined with various amounts of secondary antibody (20, 10, and 5 μ l) in checker board fashion. Cells were then analysed by flow cytometry and the minimum amount of both antibodies that gave rise to an optimum fluorescence was noted.

STAINING PROCEDURE

Prior to staining, Facs buffer [PBS with 1% (w/v) bovine serum albumine (BSA) and 0.1% (w/v) Sodium azide] was prepared and stored at 4°C. The complete content of each well of the 48 well plates (500 μ l with 5×10^5 cells) was transferred to individual labelled Falcon 2054 tubes and washed twice. Washing procedure consisted of addition of 4ml of Facs buffer, centrifugation at 1000rpm for 5min, quick inversion of the tubes to decant the supernatant and gentle resuspension (after inversion of the tubes, a volume of approximately 200 μ l of supernatant, containing the cell pellet remains in the tube). Following the second wash, 20 μ l of primary antibody was added to each cell pellet. The cells were gently resuspended in the remaining supernatant and incubated on crushed ice for 30min. Cells were washed twice in Facs buffer as before. Secondary antibody (10 μ l) was added, and the cells were resuspended as before and incubated on crushed ice for 20min. Following incubation the cells were washed twice as before and analysed by flow cytometry.

FLOW CYTOMETRY PARAMETERS

Fluorescence-activated cell sorting (FACS) is the name given to the physical separation of cells based on the presence of a cell marker specifically labelled with fluorescein, that is detected by a laser beam focused on the cells flowing as a single cell stream. Flow

cytometry is the analysis of subpopulations of cells based on fluorescence emission (as above) but without actual physical separation of the cells. A pre-set number of cells (acquired events) are analysed one by one and the intensity of fluorescence emitted by them is measured. The measurements are then plotted in a chart format (histogram) with the number of cells in the Y axis and respective level of fluorescence (measured by the photomultiplier tube 2 - PMT2) in the X axis. Prior to the analysis of samples, the cells are gated and the interval of fluorescence acquisition is set. Gating is the selection of subpopulations of cells in the sample, based on cell measurements such as cell size (measured by the forward scatter) and granularity (by the side scatter). Because virtually all cells in the samples were lymphocytes, the gating was directed to live cells. As a general rule, dead cells are smaller and more granular than live cells. The interval for measuring fluorescence was set by subtraction of the maximum level of fluorescence emitted by unstained cells and by cells labelled with an irrelevant primary antibody and a FITC-conjugated secondary antibody. The departmental FACS machine (Epics Elite) operated by Dr Linda Andrew was used to analyse samples.

3.3 Results

3.3.1 Evidence of expression of recombinant eqIFN- γ

Expression in bacteria

A modified Bradford assay (mat&meth) was used to estimate the recombinant protein recovery. Typical recovery yields, for 400 ml bacterial cultures, were approximately between 1.5 and 2mg for GST, from 1mg to 1.5mg to GSTeqIFN- γ and around 0.75mg for cleaved eqIFN- γ .

Cell lysates of bacteria expressing GST-IFN- γ fusion protein, following affinity chromatography were analysed on a SDS-PAGE gel. Coomassie blue staining of the gels (figure 3.10) reveals that each sample shows only one major protein product. The products, of approximately 28 and 43 kDa, when compared to a suitable protein size marker, correspond to the expected sizes of GST protein and GST-eqIFN- γ fusion protein, respectively. The smaller products in the lane containing GST-eqIFN- γ are possibly due to premature termination of protein synthesis or to slight proteolytic degradation of the fusion protein by *E.coli* proteases. This fusion protein product corresponds to the truncated version of eqIFN- γ (lacking signal peptide). The expression of full length eqIFN- γ protein in this system led to poor and inconsistent protein yields following purification (results not shown). Thrombin cleavage of the fusion protein reveals a major product of approximately 15 kDa, consisting of mature eqIFN- γ . In this lane can also be seen some larger products that correspond to GST tag protein not completely removed following cleavage and affinity purification.

Expression in baculovirus

Confirmation of expression of IFN- γ in the baculovirus system was obtained by plaque assay and PCR amplification of plaque viral DNA. Because wild type virus encodes the gene for β -galactosidase that is disrupted by the insertion of foreign genes, after X-gal staining, plaques corresponding to parental virus are blue, while plaques containing

baculo.eqIFN- γ are white. Figure 3.11 (A) shows a typical plaque assay of β -gal expressing parental virus. Figure 3.11 (B) depicts the PCR amplification of white plaques following one of three rounds of plaque purification. Plaque assays for eqIFN- γ recombinant virus are not shown.

Mammalian expression

Expression of eqIFN- γ in cos7 cells was assessed by northern blot analysis of total RNA preparations recovered from transfected cells harvested at different time points. A negative control consisting of total RNA derived from cells transfected with parental plasmid is also used (figure 3.12-A). Probing with IFN- γ cDNA (derived from restriction digestion of plasmid DNA) led to the detection of transcripts of approximately 500b in length, consistent with the expected size for eqIFN- γ mRNA molecules. The size of the transcripts was calculated by the relative electrophoretic distance to ribosomal RNA molecules (28S corresponding to 4718 bases and 18S to 1874) of total RNA stained with ethidium-bromide prior to blotting. A probe for the house keeping enzyme GAPDH was also used to probe RNA derived from transfections with both chimeric (figure 3.12-B) and parental plasmid (figure 3.12-C) as a measure of the quality of the RNA samples.

3.3.2 Immunodetection of recombinant eqIFN- γ

Commercially available antibodies to human and murine IFN-g were used for the detection of eqIFN- γ by western blot but no cross-reactivity was observed (see table below).

Antibody	Type	Supplier	Catalogue No.	Cross reactivity to:	
				GST-eqIFN-g	CleavedeqIFN-g
α -hum IFN- γ	mAb	Serotec	MCA1533B	no	no
α -mse IFN- γ	mAb	Serotec	MCA 1394	no	no
α -humIFN- γ	mAb	R&D	MAB 285	no	no
α -humIFN- γ	mAb	Chemicon	MAB408	no	no

This result is hardly surprising in light of the fact that the eqIFN- γ amino acid sequence as an homology of 68% towards its human counterpart and as low as 45% when compared to the murine equivalent.

Antipeptide sera

An example of the estimation of antipeptide sera sensitivity to bind corresponding immunogenic peptides is shown in table 3.1, in a typical bidimensional serial dilution of antipeptide serum against secondary antibody. In the case shown, serum V606 binds strongly to peptide 36 (adsorbed to the plate) with an endpoint of detection observed to correspond to a 1/320 dilution of the peptide serum for a 1/6400 dilution of secondary antibody. Higher dilutions of the secondary antibody did not allow an endpoint to be reached. The endpoint is calculated as half of the maximum optical density reading, in this case, at 405nm.

In the second type of ELISAS the specificity of the serum was tested by assessment of its reactivity with peptides 35 and 36. Additionally the results of the first round of ELISA are used for further optimisation of sera and secondary antibody concentrations. Assessment of serum specificity showed that sera V566 and V606 (raised against peptide 36) exhibit strong positivity in binding to peptide 36 and negligible cross reactivity with peptide 35. In the case of serum V606 (table 3.2) the endpoint was 1/400 at a secondary antibody dilution of 1/10000, confirming the results of the previous test and indicating optimal antibody concentrations of between 1/300 and 1/400 for primary antibody and around 1/10000 for secondary antibody. Comparable results were observed for serum V566 (results not shown). Additionally, pre-immune sera from both rabbits V566 and V606 showed negligible peptide binding. Sera raised against peptide 35 (V579 and V693) showed insignificant antipeptide antibody titre, demonstrating that peptide 35 has poor immunogenic potential, at least when compared with peptide 36. Sera V606 bound very poorly to GSTeqIFN- γ and to cleaved eqIFN- γ and failed to detect these proteins in western blots (results not shown).

Identification of expressed products

Anti-GSTeqIFN- γ polyclonal serum recognises not only GST and GSTeqIFN- γ fusion protein, but also thrombin cleaved eqIFN- γ in western blot (figure 3.13). Additionally, anti GSTeqIFN- γ was found to bind, in western blots, to both in house produced feline IFN- γ and commercially available human IFN- γ . These cross-reactivity studies were performed by L. McMonagle, Dept. Veterinary Pathology (results not shown).

Figures 3.14 and 3.15 show the specific detection of recombinant equine IFN- γ expressed in the baculovirus and mammalian expression backgrounds, respectively. The use of a positive control, consisting of cleaved GSTeqIFN- γ fusion protein, revealed that the baculovirus and mammalian derived eqIFN- γ have a greater molecular weight (between 20 and 30 kDa) than the *E. coli* derived eqIFN- γ .

3.3.3 Biological activity of eqIFN- γ

Cytopathic effect reduction assay

Results shown in figure 3.16 show typical assays for the determination of the level of protection conferred by recombinant eqIFN- γ to the challenge of MDBK cells with VSV virus. The eqIFN- γ antiviral potency was calculated as the reciprocal of the dilution that gives rise to protection of 50% of the wells, which is the potency in laboratory units of the sample (in this case 100 μ l). For calculation of the potency per millilitre, the result is multiplied by the dilution factor (in this case 10). The titres of the individual IFN- γ preparations (table 3.3), expressed in laboratory units (U), are as follows: 640U for BacIFN72; no protection for BacIFN96; 2560U for PciIFN24; 640U for PciIFN48 and no protection for Bacwt and Pcineo.

Earlier experiments that made use of an equine cell line (EEK) in this assay system showed that, baculovirus derived eqIFN- γ , protected cells from challenge virus (at 100TCID₅₀) up to a dilution of 10⁻⁴, corresponding to 100000 laboratory units of eqIFN- γ per ml (results not shown).

PBL proliferation assay

Equine peripheral blood lymphocytes were stimulated with mitogen, eqIFN- γ or both and their proliferative response was assessed by the relative amount of tritiated thymidine incorporated as the cells divide. Experiments were conducted in triplicate and the average ^3H -thymidine incorporation, measured in counts per minute (cpm), and standard deviation was calculated.

As a means to investigate if equine peripheral blood lymphocytes would proliferate in response to mitogen, varying concentrations of concanavalin A (ConA standard curve), were used to stimulate the cells. Decreasing quantities of ConA corresponded to a decrease in the average ^3H -thymidine incorporation in a linear, dose dependent manner. Incorporation varied from 13480 cpm, corresponding to 5 $\mu\text{g}/\text{ml}$ of ConA, to a value of 2488 cpm, for 0.16 $\mu\text{g}/\text{ml}$. Also, background levels of incorporation in wells containing unstimulated cells were 1984 cpm and in wells with no cells 30 cpm (table 3.4 and figure 3.17).

Cells stimulated with BacIFN72 alone, exhibited a mild dose response pattern of incorporation, varying from a maximum of 6541 cpm, for a $\frac{1}{2}$ dilution of IFN- γ , to a minimum of 2813 cpm, corresponding to a 1/256 dilution, and comparable to the basal incorporation observed in cells stimulated with the negative control (Bacwt) of approximately 2200 cpm (table 3.5 and figure 3.18).

When cells treated with BacIFN72 were co-stimulated with 0.3 $\mu\text{g}/\text{ml}$ of ConA, a maximum incorporation of 64896 cpm decreased linearly to a minimum of 2863 cpm in the same dilution interval. Significantly, the basal incorporation in response to Bacwt plus 0.3mg/ml of ConA suffered little or no effect, remaining at the low level of approximately 2800 cpm (table 3.6 and figure 3.19). The difference in proliferation between cells stimulated with eqIFN- γ alone or eqIFN- γ plus mitogen represents a striking ten fold increase in the level of ^3H - thymidine incorporation. A comparison of incorporation levels between the different samples is shown in figure 3.20.

Supernatants corresponding to BacIFN96 were also tested using this assay system but no correlation between dilution of the sample and cell proliferation could be extrapolated.

MHC class II upregulation

Unstimulated equine “T cells” were labelled with antibodies for a number of cell surface molecules and analysed by flow cytometry to investigate the relative frequency of lymphocyte subsets in the cell preparation. The results obtained are within the typical ranges described by other groups (Kydd *et al*, 1991; Lunn *et al*, 1997), where anti-B cells antibody (an homologue of CD19) bound to 3.5% of the cells; anti-CD5 bound to 85.5% and anti-CD4 to 60% (figure 3.21).

Two experiments were designed to investigate if recombinant eqIFN- γ would upregulate MHC class II molecules on the surface of the “T cells”. In the first, cells were stimulated with eqIFN- γ preparations alone and in the second cells were co-stimulated with human recombinant IL-2.

In the first set of experiments, following incubation, there was a great number of dead cells, on both stimulated and unstimulated samples, as assessed by size and granularity (figure 3.22 A and C). Also, staining with a-MHC II (figure 3.22 D) revealed a bimodal pattern of fluorescence where some T cells stain brighter while others stain dimmer, depending principally in their activation state. Within the bright population is possibly the entire population of B cells (Rideout *et al*, 1990). This pattern was maintained in all samples. However, increasing concentrations of eqIFN- γ in this experiment, led to an increase in the number of dim cells expressing MHC class II molecules while the brighter population of cells remained largely unaltered (figure 3.23 A to D). A total of three experiments were performed with similar results.

In the second set of experiments, cells were incubated with eqIFN- γ and co-stimulated with recombinant human IL-2. In this case, the number of dead cells was much smaller (figure 3.24 A and C). More importantly, although the bimodal curve of MHC II staining was maintained, it was the brighter cells that responded to the presence of IFN- γ with an

increased expression of the surface molecule with the dim cells remaining mostly unaltered (figure 3.25 A to E). Once again three independent experiments were performed with similar results.

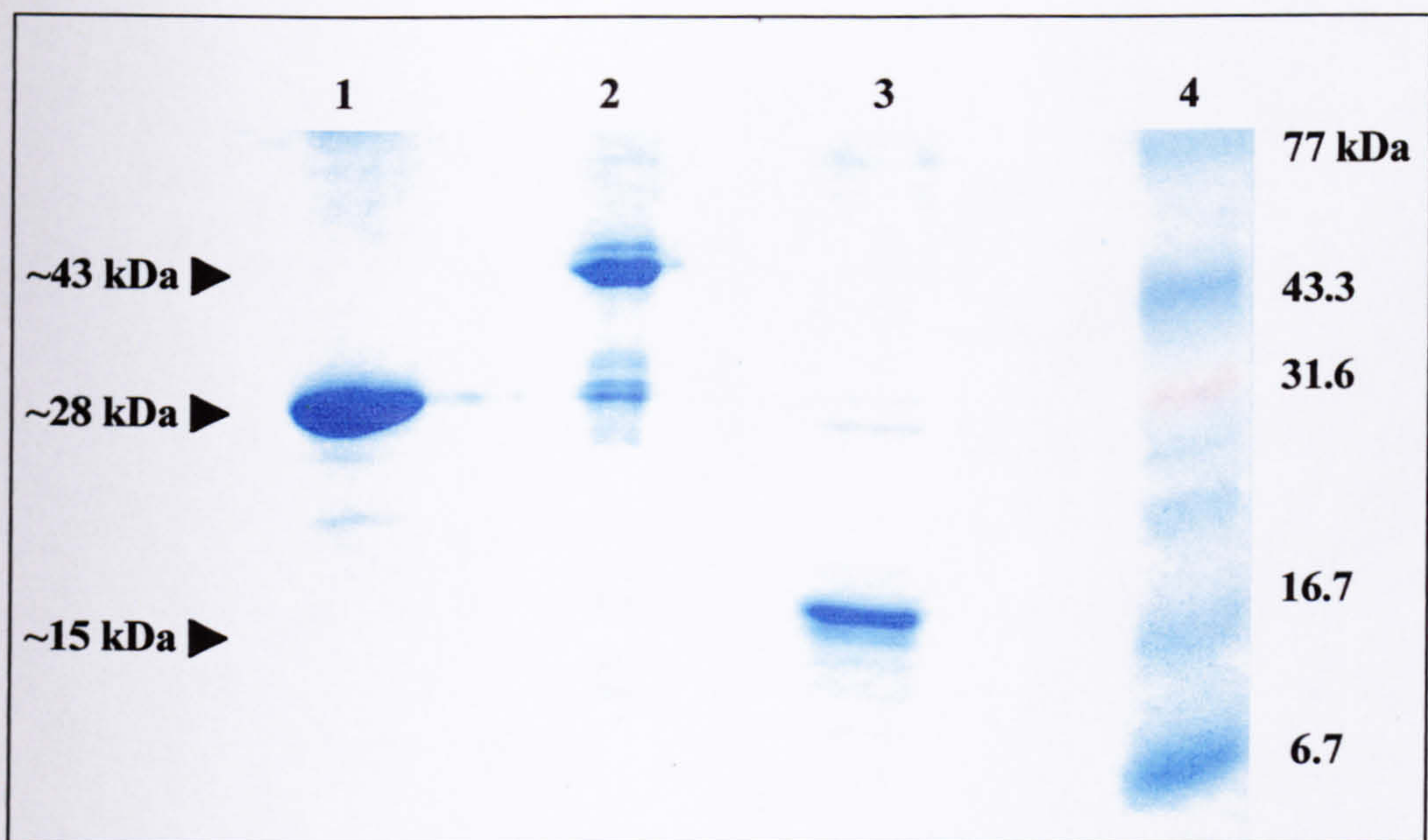


Figure 3.10 Expression of equine IFN- γ in *E. coli*.

Affinity purified protein products of bacteria expressing GST and GSTeqIFN- γ , were analysed by SDS-PAGE and stained with Coomassie blue. Cleaved GSTeqIFN γ was also analysed. Approximately 10 μ g of each protein preparation was loaded on a 15% gel as follows: Lane1- GST; Lane2- GSTeqIFN- γ fusion; lane3- cleaved eqIFN- γ . On lane 4 was loaded 10 μ l of protein standard.

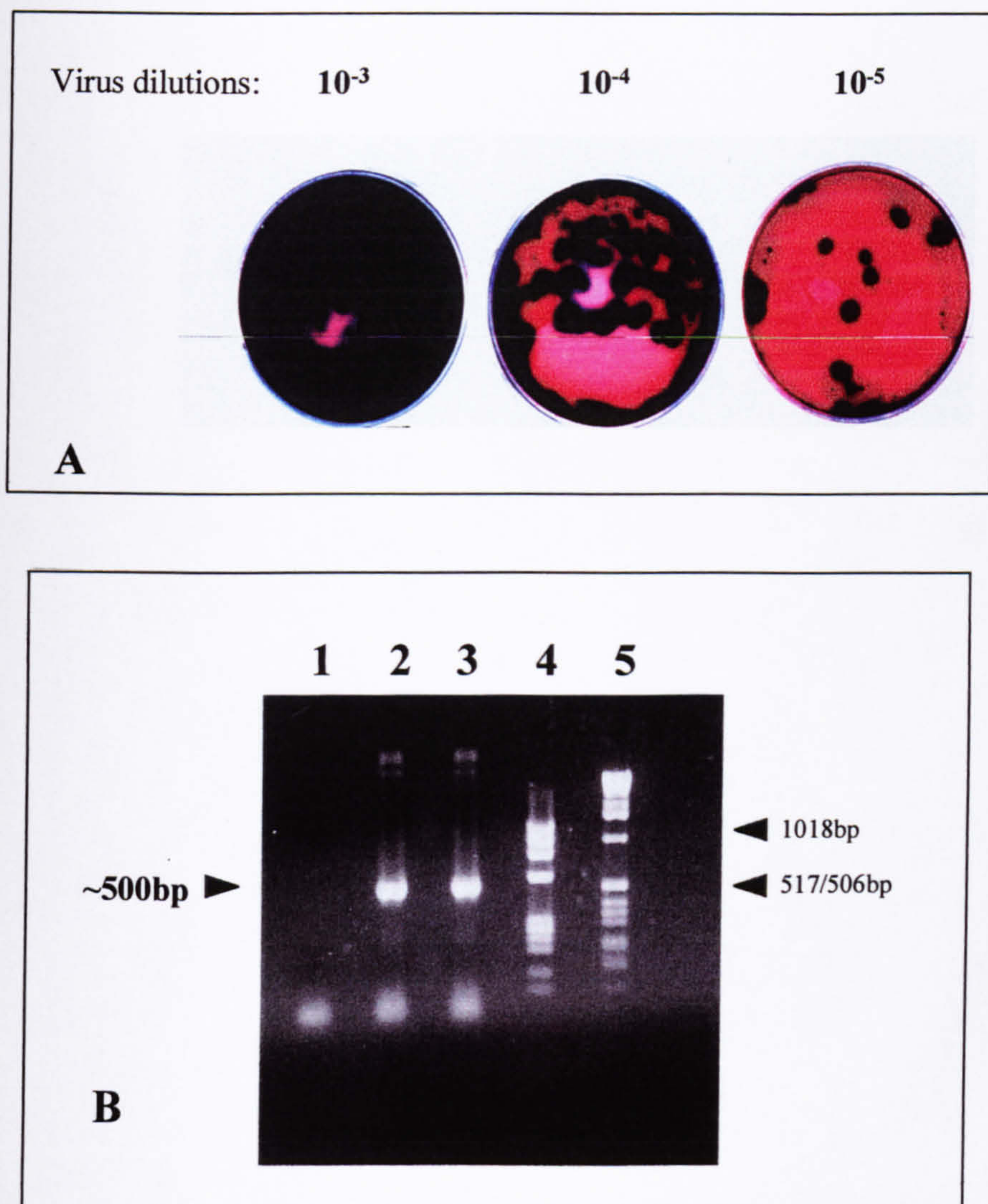


Figure 3.11 A- Typical plaque assay of baculovirus wild type.

Cells were stained with X-gal for blue plaque development. At the 10^{-5} virus dilution, 9 plaques can clearly be counted.

B- PCR amplification of Baculo.eqIFN- γ plaque picks.

Plaque DNA amplified with equine IFN- γ specific primers, was loaded on a 1.2% TAE agarose gel as follows: Lanes-1, 2 and 3, were loaded with 10 μ l of individual PCR products; Lane 4-1 μ g of ϕ X 174 RF DNA/HaeIII fragments; Lane 5-1 μ g of 1Kb DNA ladder. A band of approximately 500bp was obtained for positive clones (lanes 2 and 3).

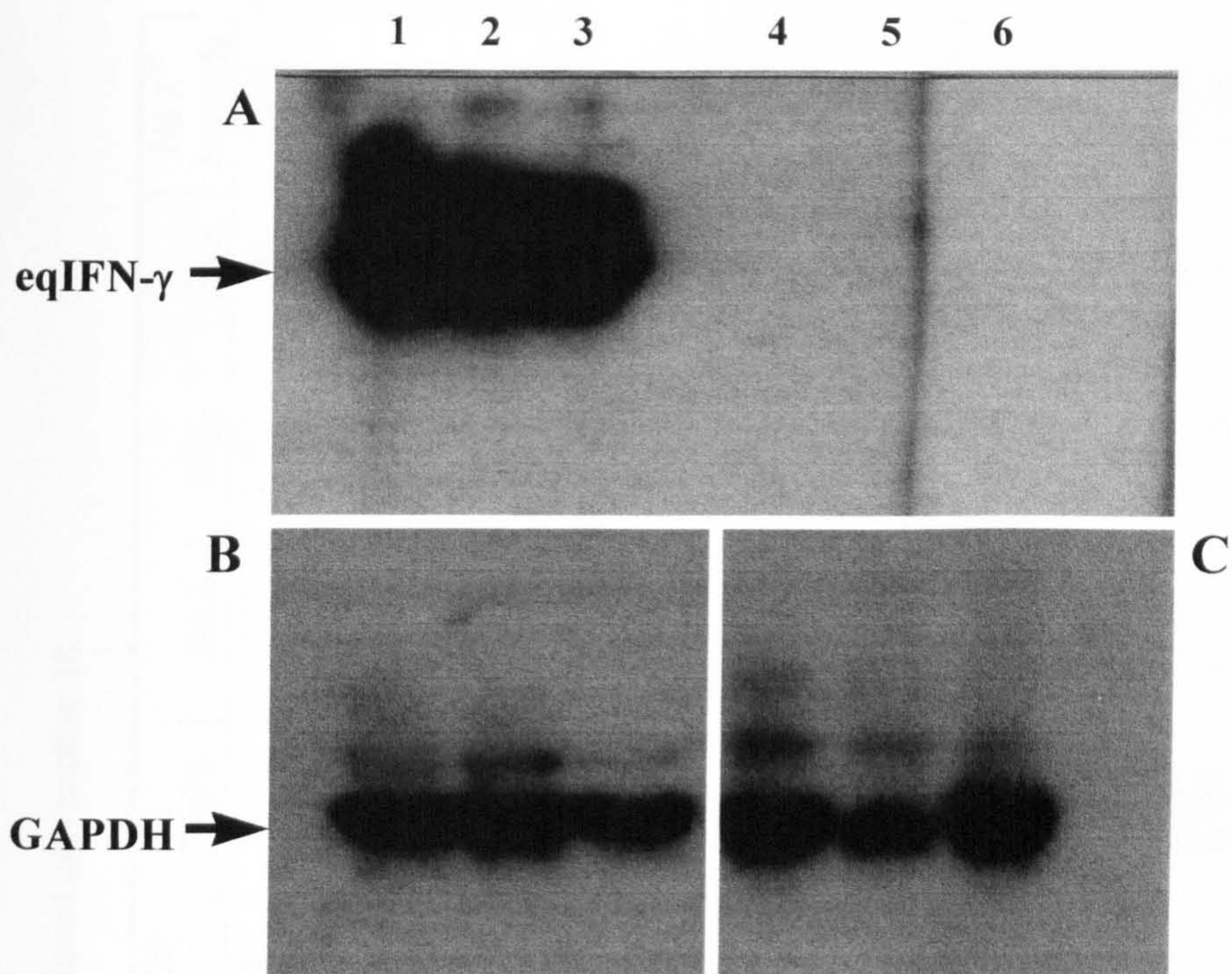


Figure 3.12 Expression of equine IFN- γ in mammalian cells.

A- RNA derived from transfected cos7 cells was probed with an IFN- γ gene specific probe. RNA (15 μ g/lane) was loaded as follows: Lanes 1, 2 and 3 pCI-eqIFN- γ at 24, 48 and 72 hours post-transfection, respectively; Lanes 4, 5 and 6 - pCI-neo parental plasmid at 24, 48 and 72 hours post-transfection, respectively.

B and C - The same samples as in gel A, loaded in the same order, were probed with a GAPDH probe. Gels B and C were sized to fit gel A alignment.

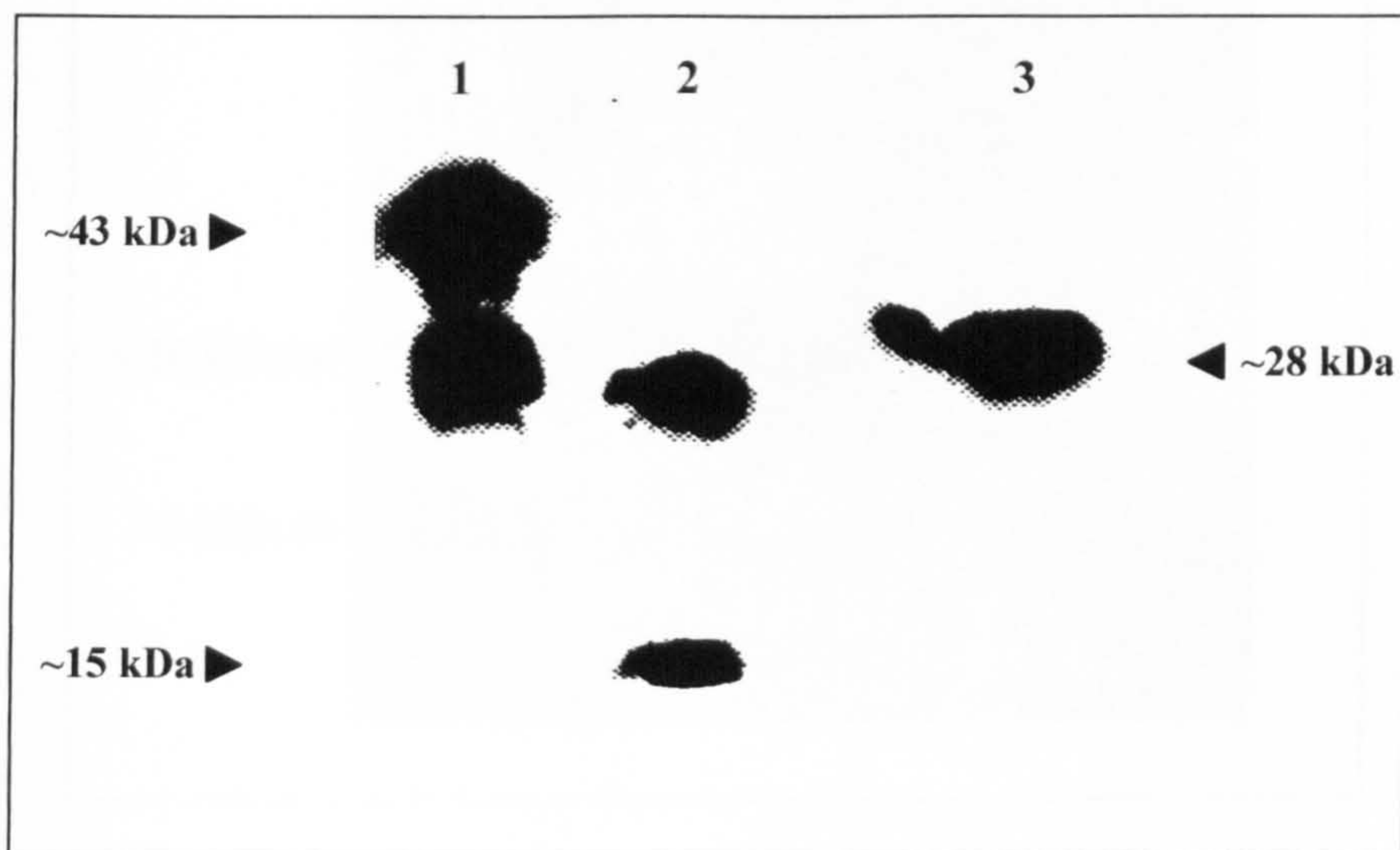


Figure 3.13 Specificity of anti-GSTeqIFN- γ serum.

Samples were loaded in a 12% SDS-PAGE gel as follows: Lane1- GSTeqIFN- γ (5 μ g); lane2- cleaved GSTeqIFN- γ (5 μ g); lane3- GST (2 μ g).

Protein quantities were assessed by Bradford assay. Detection using primary antibody (anti-GSTeqIFN- γ) at a 1/10000 dilution followed by secondary antibody (HRP conjugated goat anti-rabbit IgG polyclonal antibody) diluted 1/5000. Blot developed using ECL reagents. Film exposed for 15 seconds.

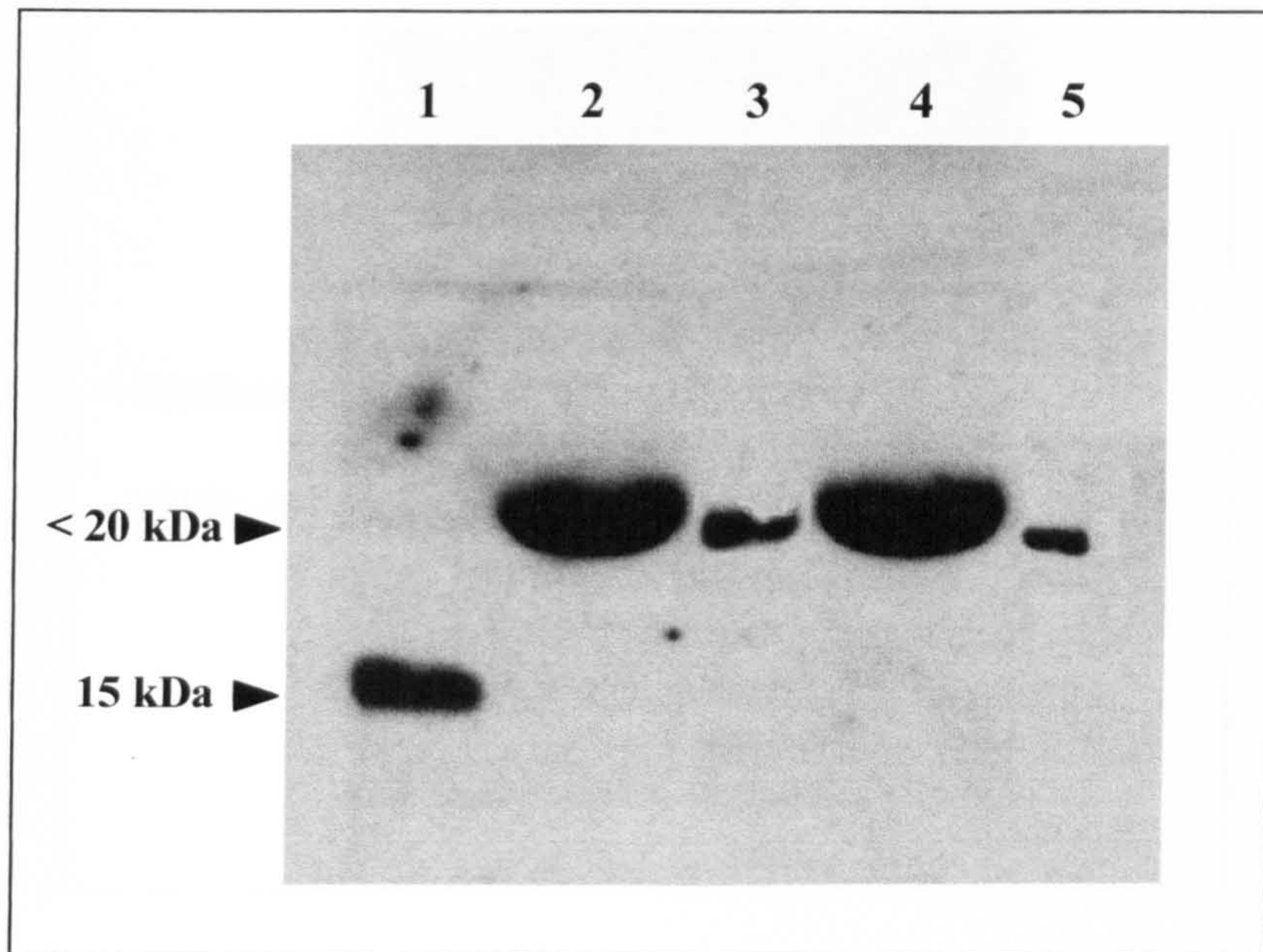


Figure 3.14 Identification of eqIFN- γ expressed in baculovirus.

Samples were loaded in a 15% SDS-PAGE gel as follows: lane1- cleaved GSTeqIFN- γ positive control (2 μ g); lane2- 72 hours baculoeqIFN- γ infection supernatant (10 μ l); lane3- 72 hours baculoeqIFN- γ infection cell lysate(10 μ l); lane4- 96 hours baculoeqIFN- γ infection supernatant(10 μ l); lane5- 96 hours baculoeqIFN- γ infection cell lysate(10 μ l).Detection using primary antibody (anti-GSTeqIFN- γ) at a 1/10000 dilution followed by secondary antibody (HRP conjugated goat anti-rabbit IgG polyclonal antibody) diluted 1/5000. Blot developed using ECL reagents. Film exposed for 1 minute.

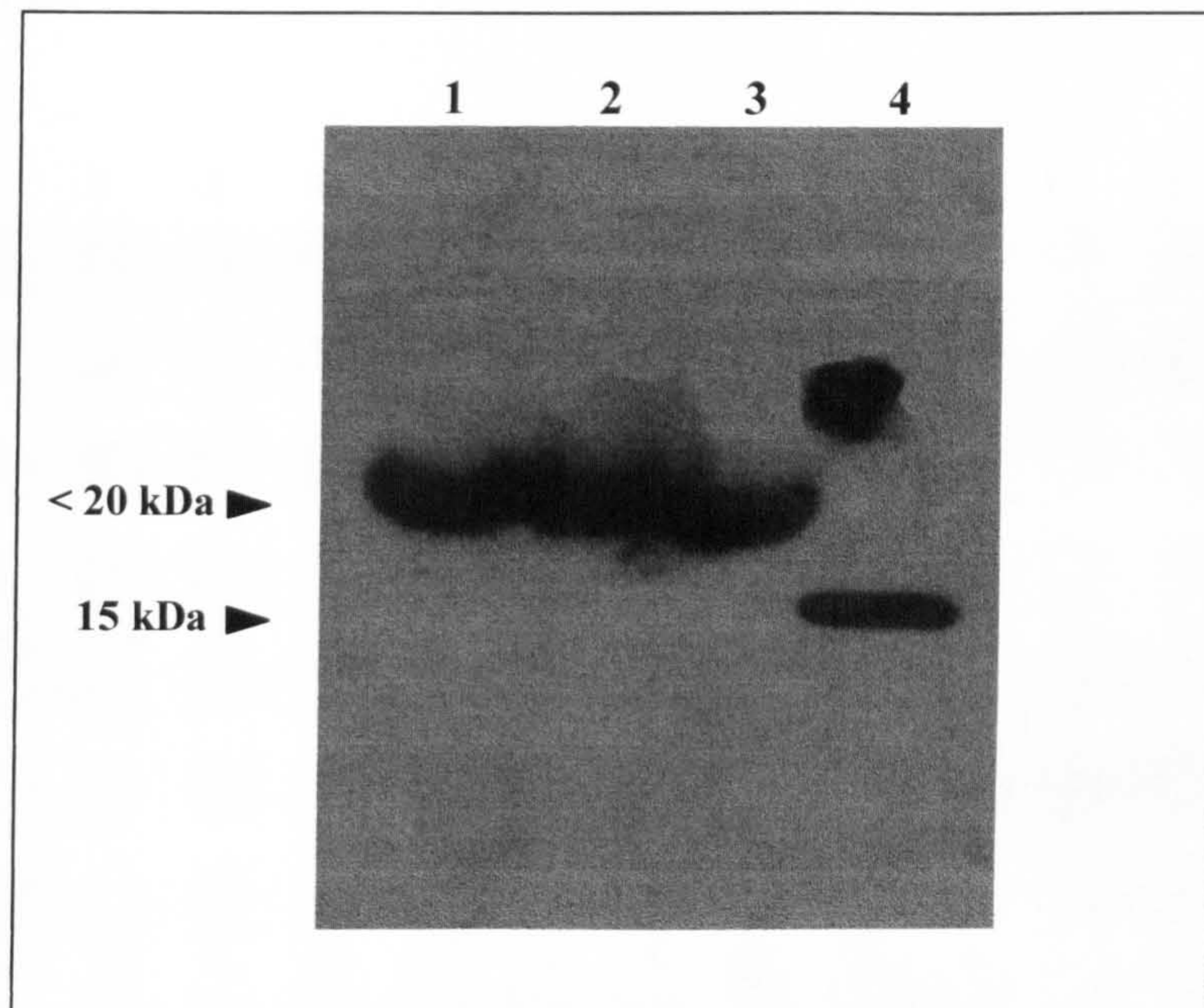


Figure 3.15 Identification of eqIFN- γ expressed in mammalian cells (cos7).

Samples (10 μ l each) were loaded in a 12% SDS-PAGE gel as follows: Lane1, 2, 3 pCI-neo.eqIFN- γ transfection supernatant of 24, 48 and 72 hours, respectively; lane4-cleaved GSTeqIFN- γ positive control (2 μ g). Detection using primary antibody (anti-GSTeqIFN γ) at 1/10000 dilution followed by secondary antibody (HRP conjugated goat anti-rabbit IgG polyclonal antibody) diluted 1/5000. Blot developed using ECL reagents. Film exposed for 5 minutes.

A- BacIFN72

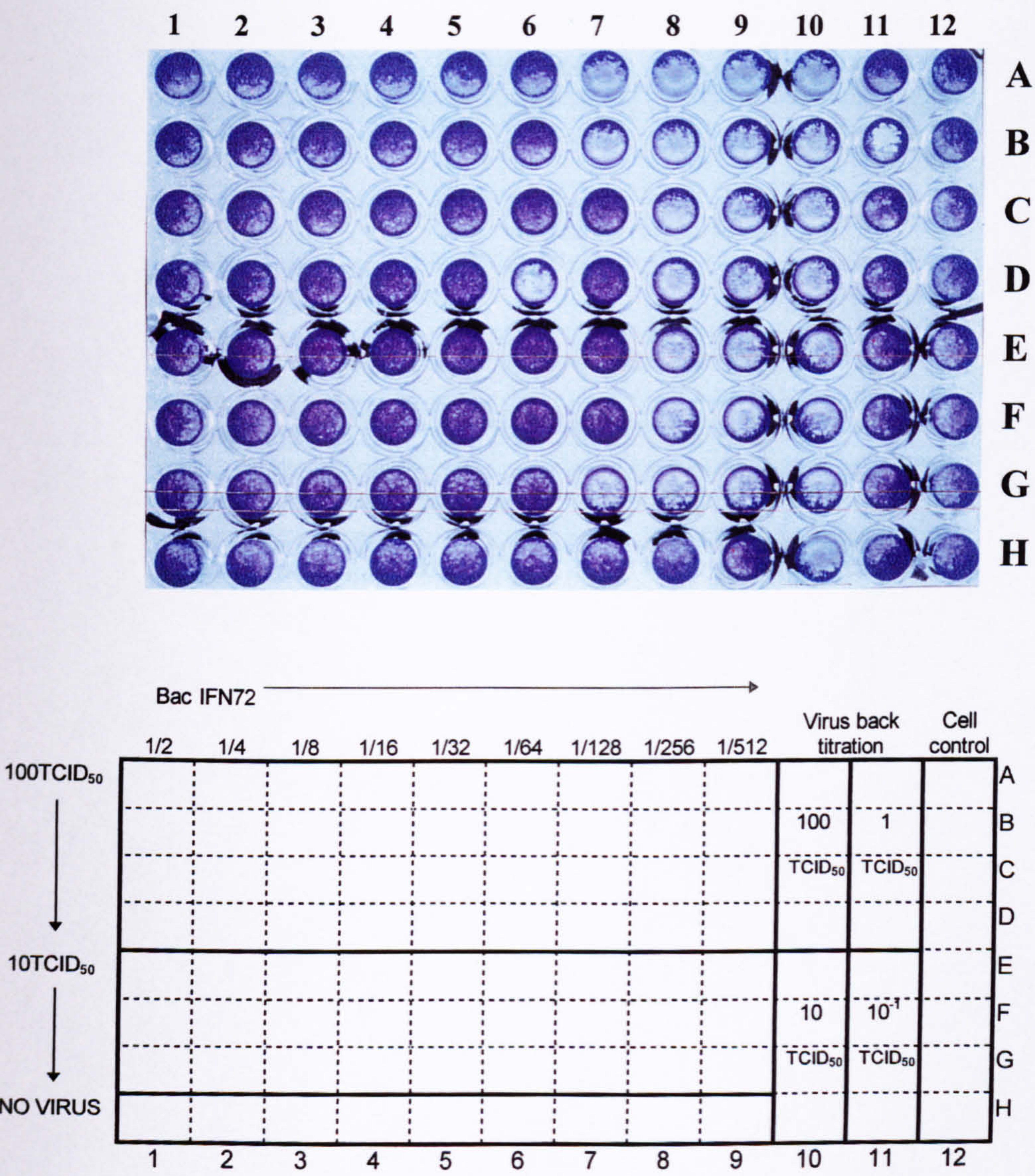


Figure 3.16 Cytopathic effect reduction assay (continued overleaf).

B- PciIFN24

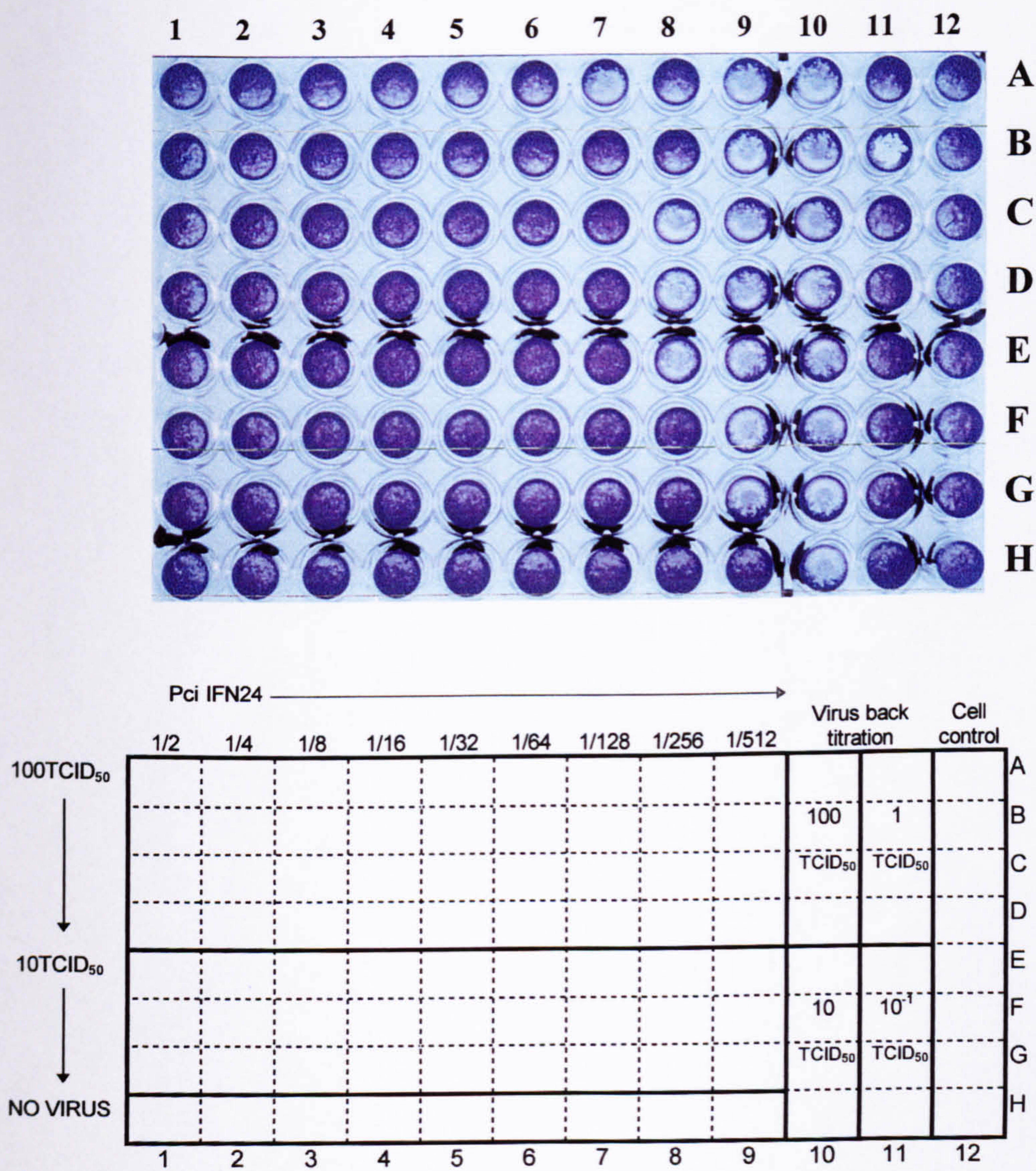


Figure 3.16 Cytopathic effect reduction assay (continued overleaf).

C- Pcineo / Bacwt

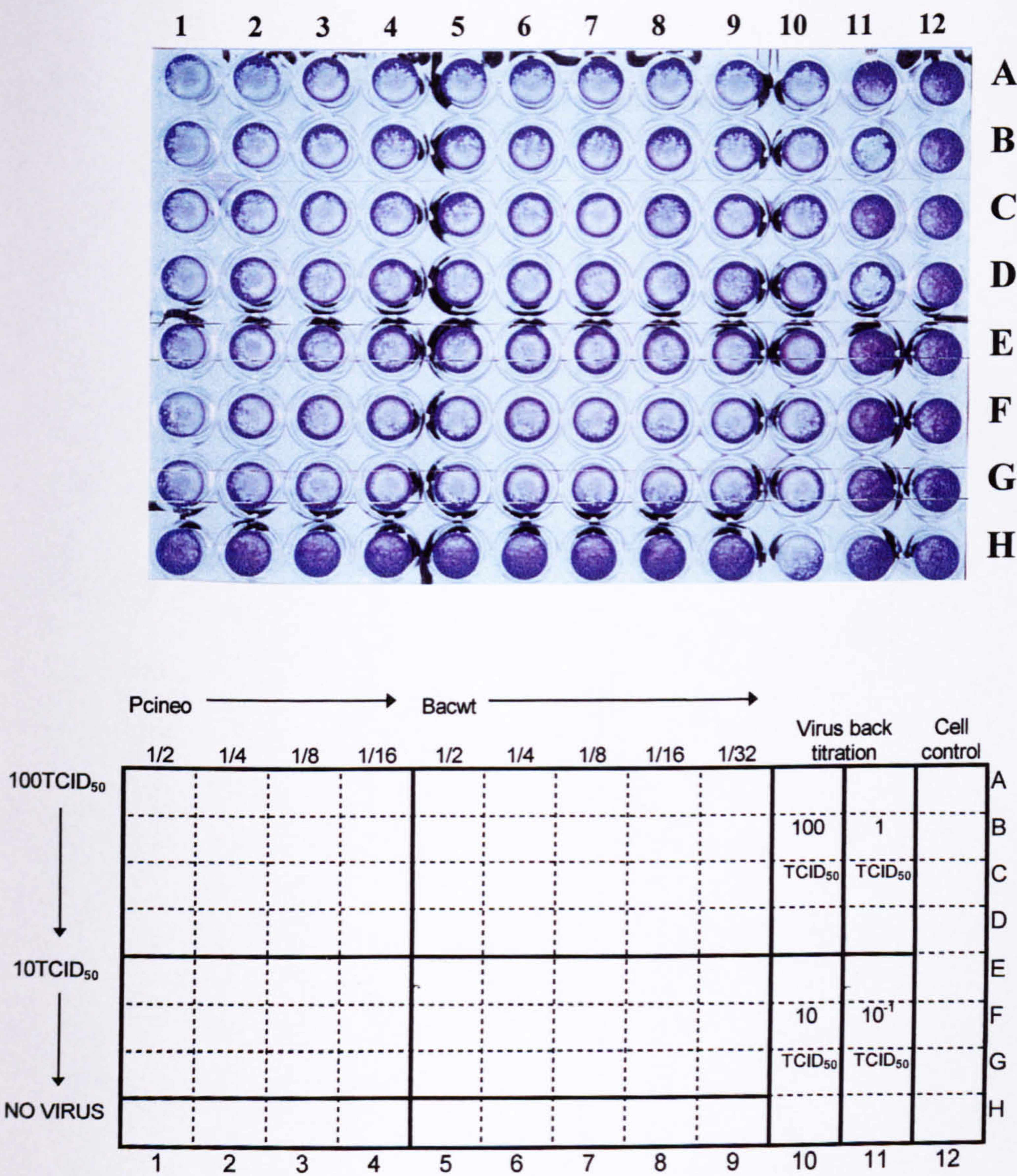


Figure 3.16 Cytopathic effect reduction assay. MDBK (1.5X10⁴ cells per well) were stimulated with twofold dilutions of eqIFN- γ preparations or parental vector controls and challenged with VSV. Each sample was tested in an individual plate. Plate A - bacIFN72; Plate B - PciIFN24; Plate C -columns 1 to 5 contain twofold dilutions of BacWT and columns 6 to 9 Pcineo. Conditions are detailed on correspondent charts.

Table 3.3 Summary of CPE reduction assay results

EqIFN-γ preparation	Dilution	% Protection	Titre
BacIFN72	1/32	100%	640U/ml
	1/64	50%	
	1/128	0	
BacIFN96	All	0	-
Bacwt	All	0	-
PciIFN24	1/64	100%	2560U/ml
	1/128	75%	
	1/256	50%	
	1/512	0	
PciIFN48	1/32	100%	640U/ml
	1/64	50%	
	1/128	0	
Pcineo	All	0	-
BacIFN 1.2.1	1/10000	100%	10 ⁶ U/ml
	1/100000	50%	
	1/1000000	0	
BacIFN 1.3.1	1/10000	100%	~5x10 ⁵ U/ml
	1/100000	25%	
	1/1000000	0	
Bacwt	All	0	

Virus challenge was 100TCID₅₀ per well with development of full CPE, in control wells, within 48 hours. BacIFN 1.2.1 and 1.3.1 are two further baculovirus expressing eqIFN-γ developed early in the project and shown, at the time, to have a potent antiviral effect.

Table 3.4 Proliferation of equine PBLs in response to ConA, measured by ³H-thymidine incorporation. Values represent scintillation counts per minute (cpm). The average and standard deviation (ST. Dev.) corresponding to each dilution are also shown

ConA standard curve					
Dilutions	Counts per minute (CPM) X3			Average	ST.Dev.
5mg/ml	13264.9	14237.5	12938.5	13480.3	675.8
2.5mg/ml	8307.3	7676.3	7372.2	7785.3	477.0
1.25mg/ml	5432.3	5075.2	4918.9	5142.1	263.2
0.62mg/ml	3575.3	3645.4	3930.4	3717.0	188.1
0.31mg/ml	2636.9	3069.9	3146.8	2951.2	274.9
0.16mg/ml	2694.7	2484.8	2284.4	2487.9	205.2
0	2119.7	2108.1	1724.2	1984.0	225.7
No cells	22.0	46.2	22.3	30.2	13.9

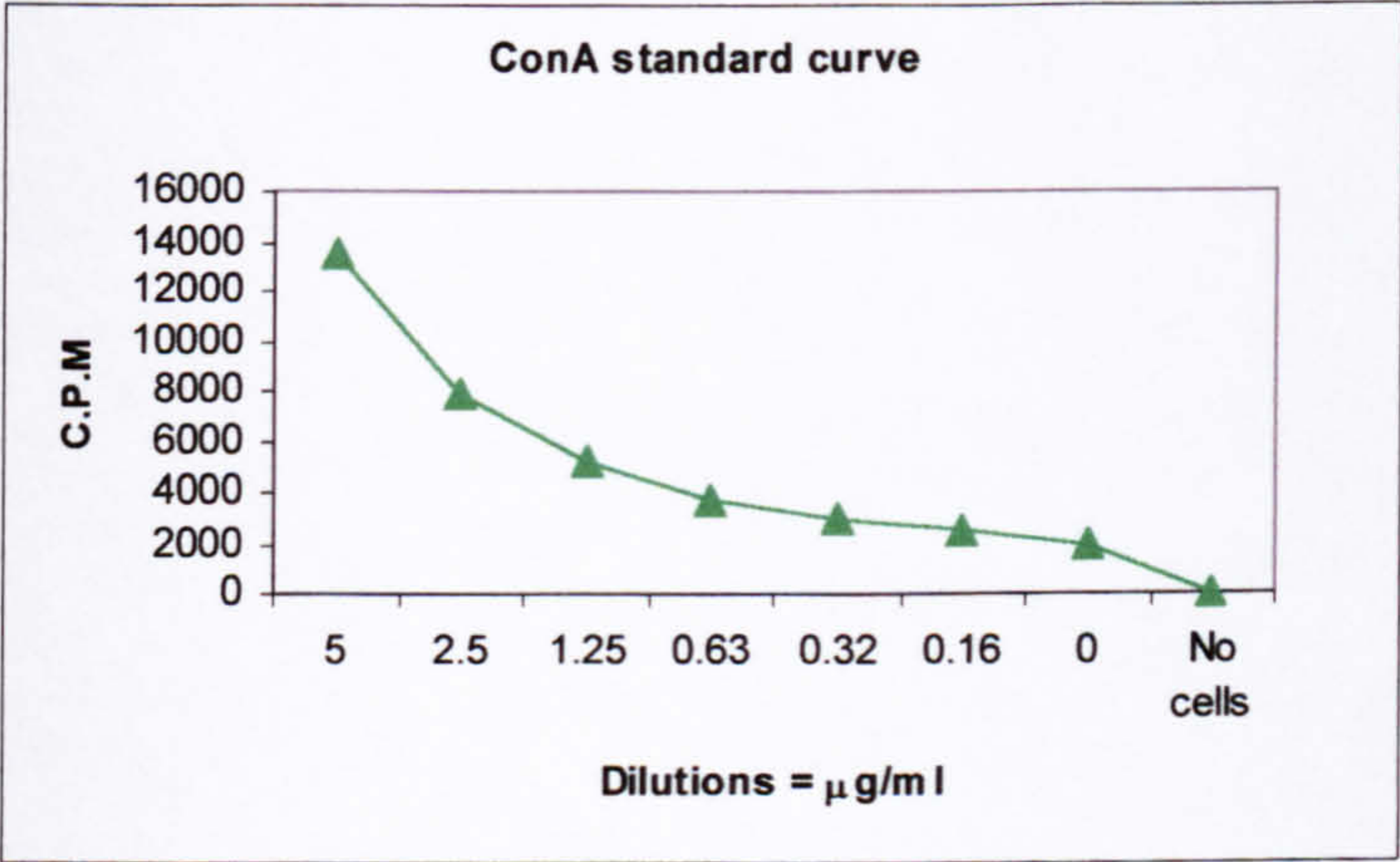


Figure 3.17 Graphical representation of averages depicted in table 3.4.

Table 3.5 Proliferation of equine PBLs in response to BacIFN72, measured by ³H-thymidine incorporation as before.

BaculoIFN					
Dilutions	Counts per minute (CPM) X3			Average	ST.Dev.
1/2	6265.8	6950.1	6409.8	6541.9	360.7
1/4	5968.9	6084.6	6295.2	6116.2	165.4
1/8	5231.5	5127.1	5371.4	5243.3	122.6
1/16	4792.0	4273.7	4471.4	4512.4	261.6
1/32	3724.9	4094.0	3918.3	3912.4	184.6
1/64	3410.0	3864.2	3718.1	3664.1	231.9
1/128	2646.9	2767.8	3026.6	2813.7	193.9
1/256	2503.2	3112.5	2736.7	2784.1	307.4
Baculo WT					
Dilutions	Counts per minute (CPM) X3			Average	ST.Dev.
1/2	2962.2	2925.2	2749.3	2878.9	113.7
1/4	1800.3	2239.8	1797.8	1946.0	254.5
1/8	2494.4	2118.7	1945.6	2186.3	280.6
1/16	2022.9	2064.8	2347.2	2144.9	176.4

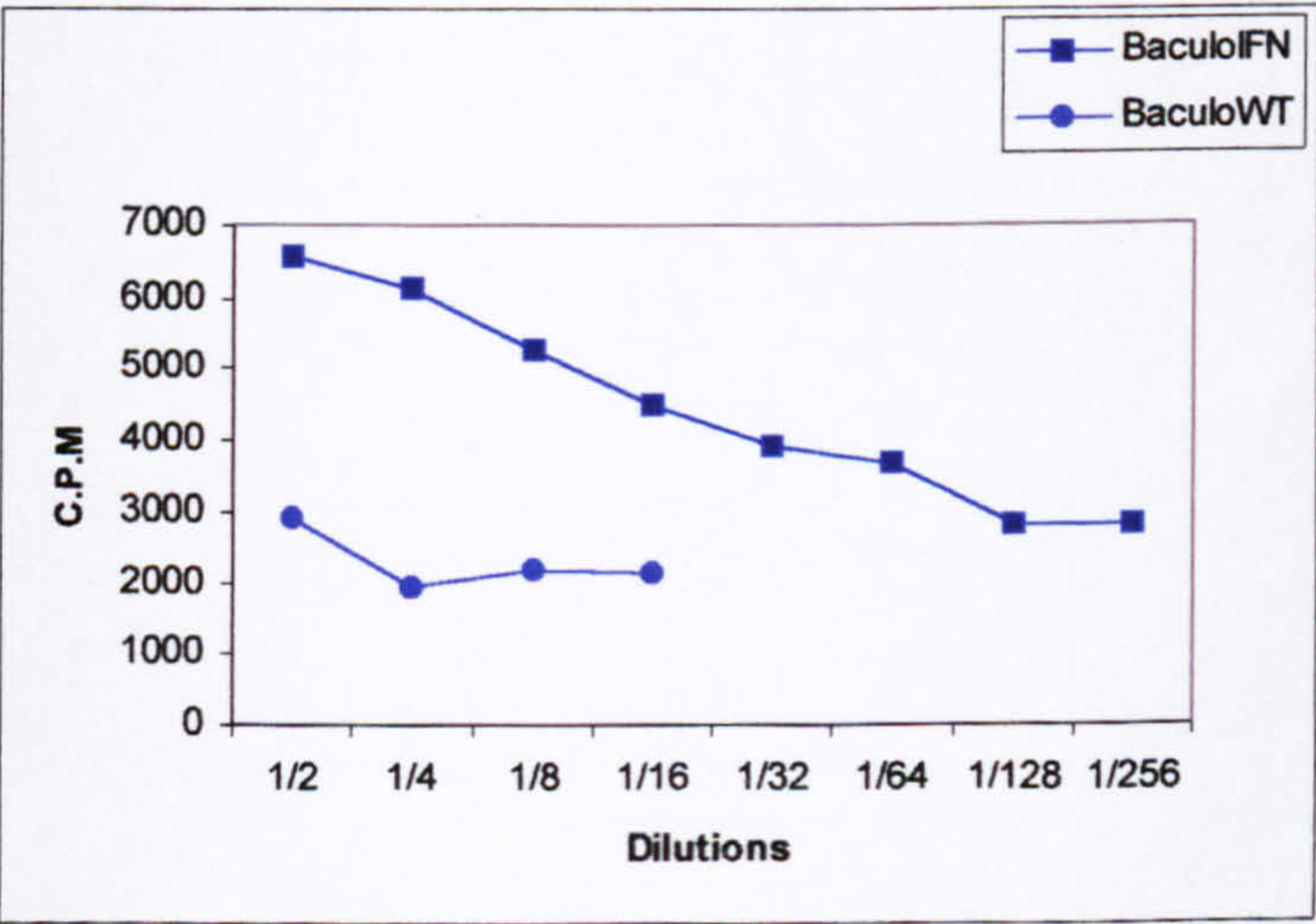


Figure 3.18 Graphical representation of averages depicted in table 3.5.

Table 3.6 Proliferation of equine PBLs in response to BacIFN72 + ConA, measured by ³H-thymidine incorporation as before.

BaculoIFN + ConA @ 0.3µg/ml					
Dilutions	Counts per minute (CPM) X3			Average	ST.Dev.
1/2	64184.2	66218.1	64287.3	64896.5	1145.7
1/4	47233.0	45519.6	44204.9	45652.5	1518.4
1/8	21596.2	20952.0	21704.1	21417.4	406.7
1/16	10187.2	12306.8	14057.6	12183.9	1938.1
1/32	6308.1	6382.1	6410.2	6366.8	52.7
1/64	4128.5	4244.7	3815.4	4062.9	222.0
1/128	3472.9	3548.8	3117.4	3379.7	230.3
1/256	2940.6	2941.9	2706.6	2863.0	135.5
Baculo WT + ConA @ 0.3µg/ml					
Dilutions	Counts per minute (CPM) X3			Average	ST.Dev.
1/2	3086.7	2847.0	2771.9	2901.9	190.1
1/4	3462.8	3191.0	3069.7	3241.2	201.3
1/8	2671.6	2803.6	2472.9	2649.4	166.5
1/16	2937.0	2644.0	2734.1	2771.7	150.1

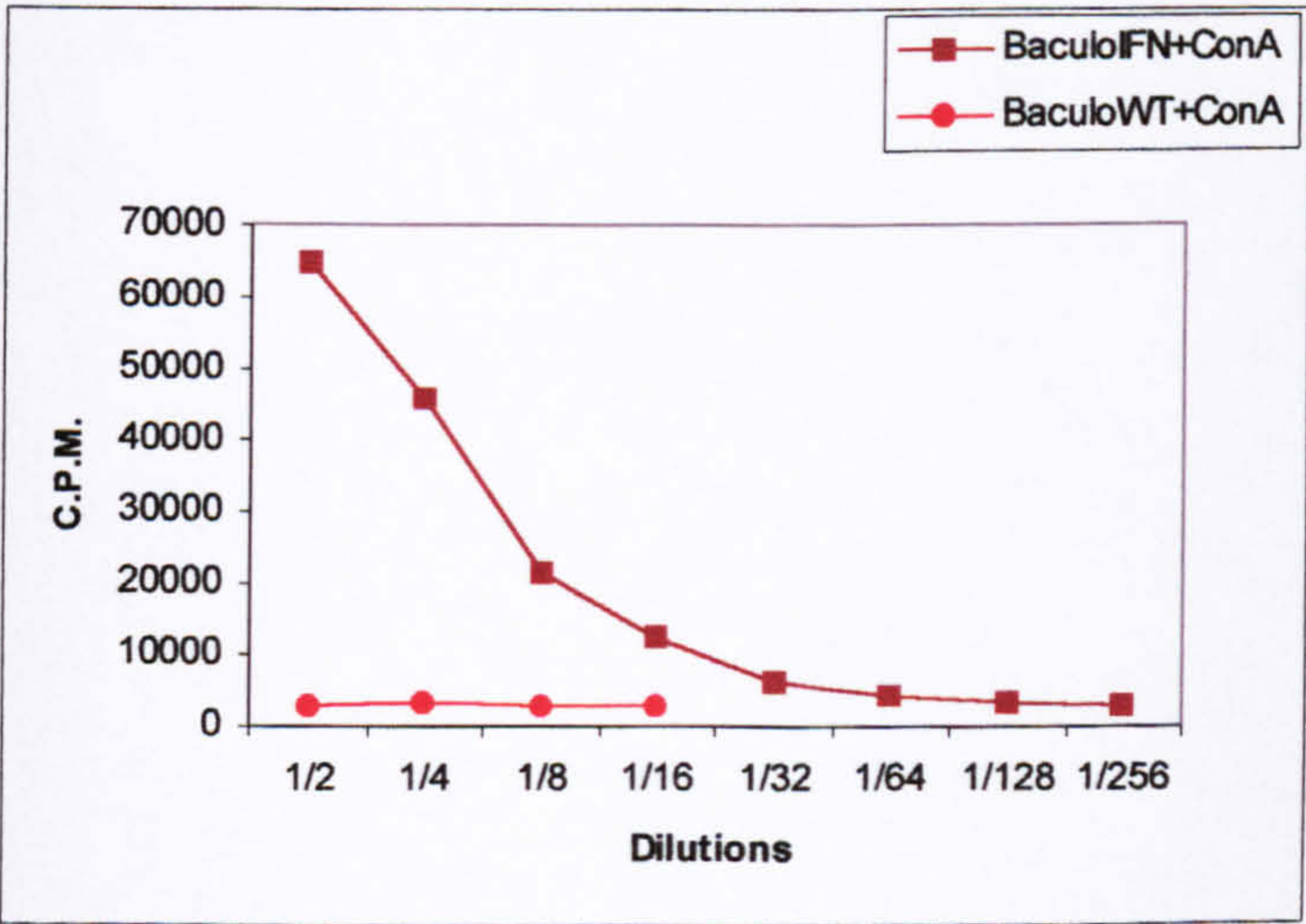


Figure 3.19 Graphical representation of averages depicted in table 3.6.

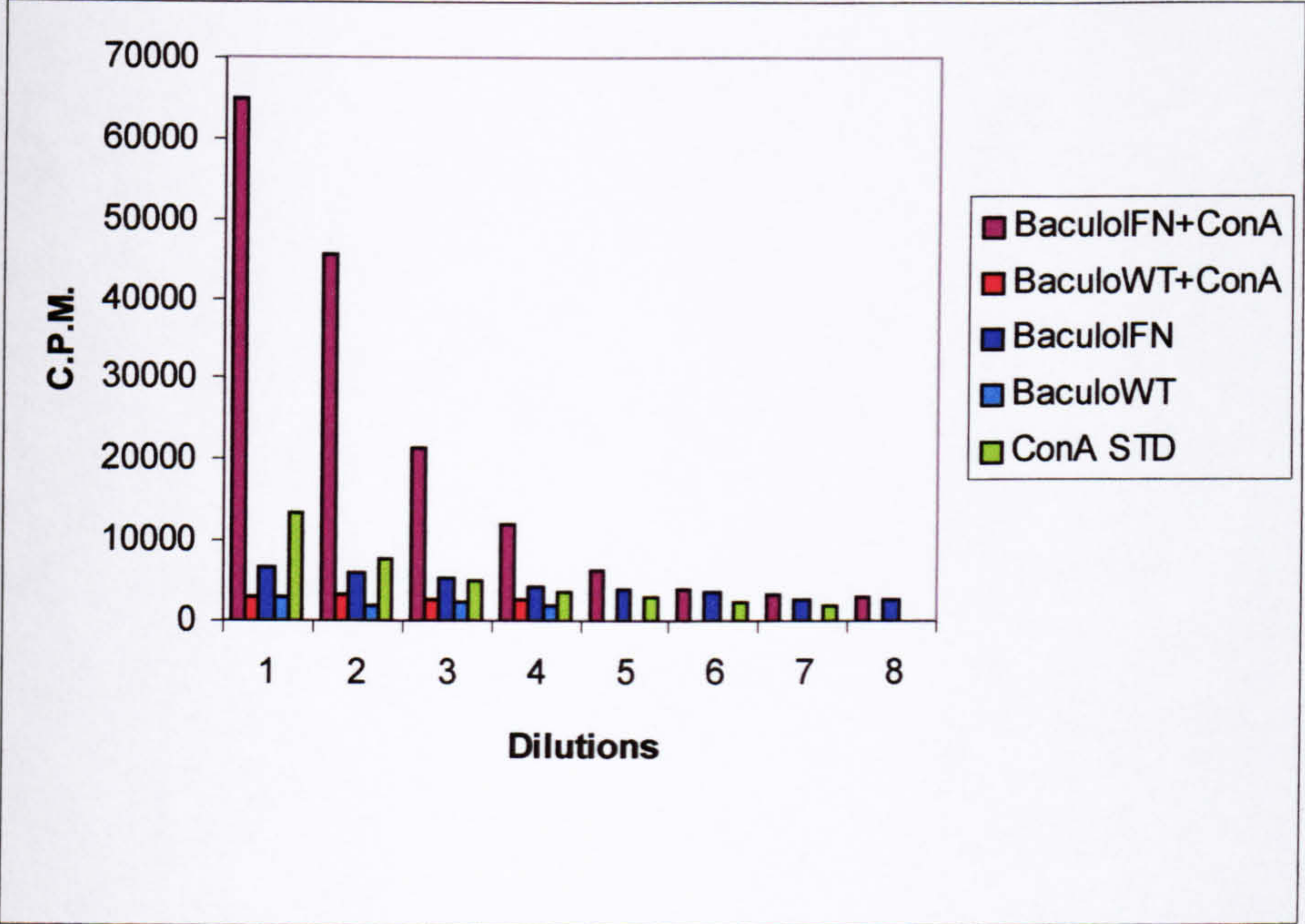


Figure 3.20 Comparison of results shown in figures 3.17, 3.18, 3.19.

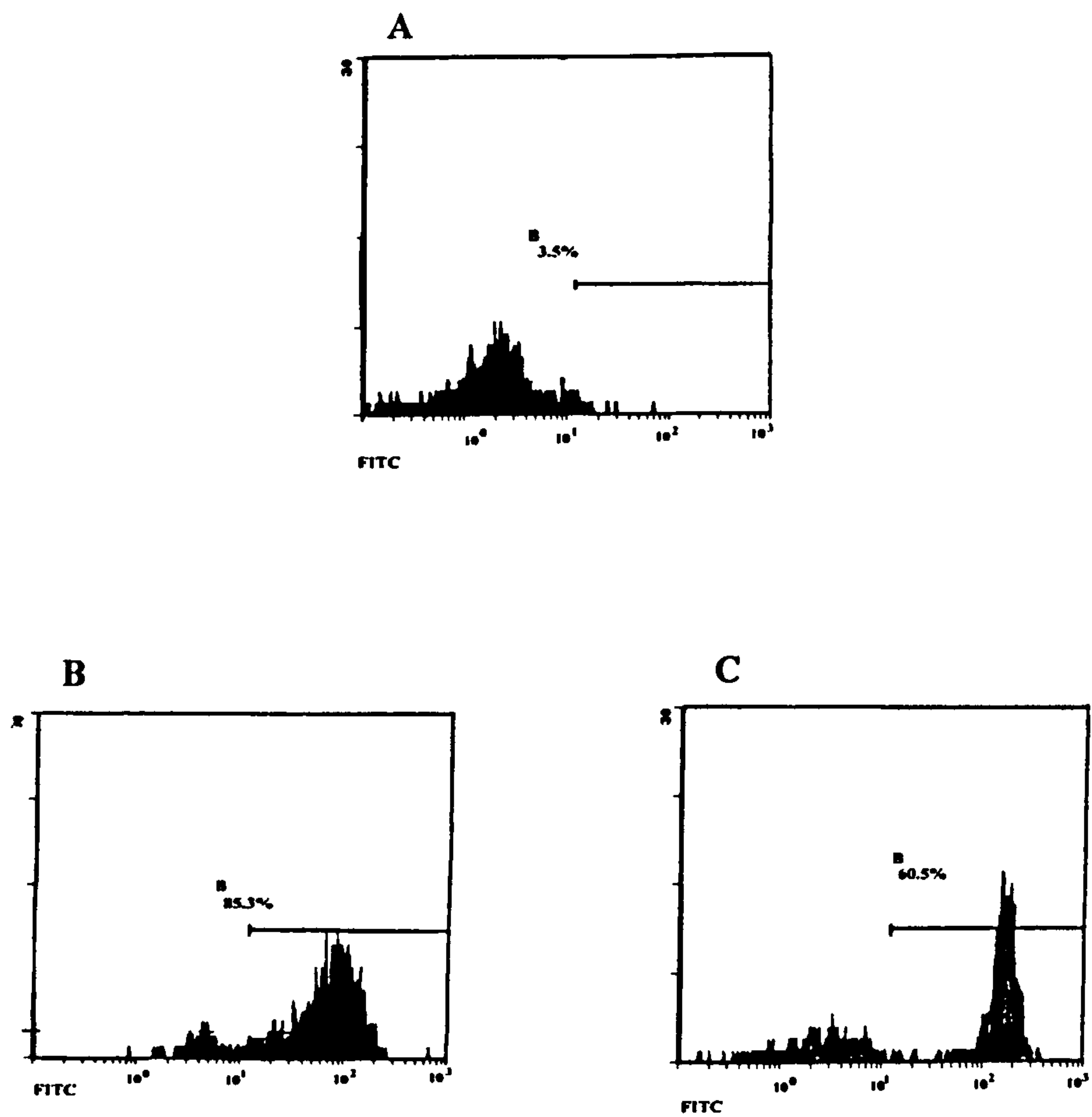


Figure 3.21 Frequency of lymphocyte subpopulations in enriched equine T cell preparations. Cells were labelled with the primary antibody: α -equine Bcell (A); α -equine CD5 (B) and α -equine CD4 (C) and with α -mouse:FITC as secondary antibody. Gating was set for live cells, based on cell granularity.

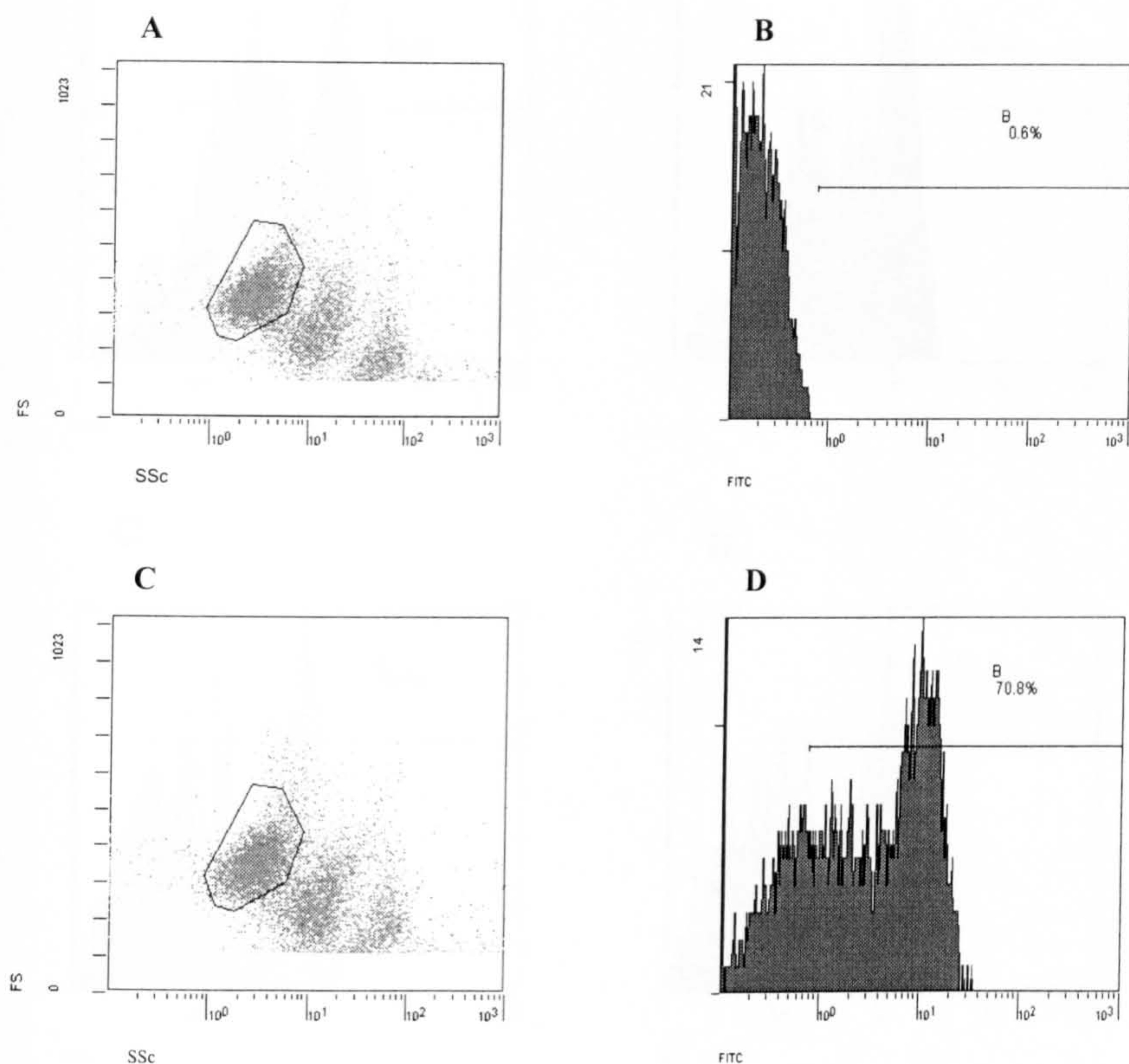


Figure 3.22 Flow cytometry analysis setup (experiment 1).

Gating of lymphocytes using forward and side scatter (A and C) and setting of fluorescence acquisition interval using orthogonal scatter and PMT2 (B and D). A and B are unstimulated cells labelled with isotypic antibody (α -gq24 / α -mouse:FITC) and C and D are unstimulated cells labelled with α -MHCII / α -mouse:FITC

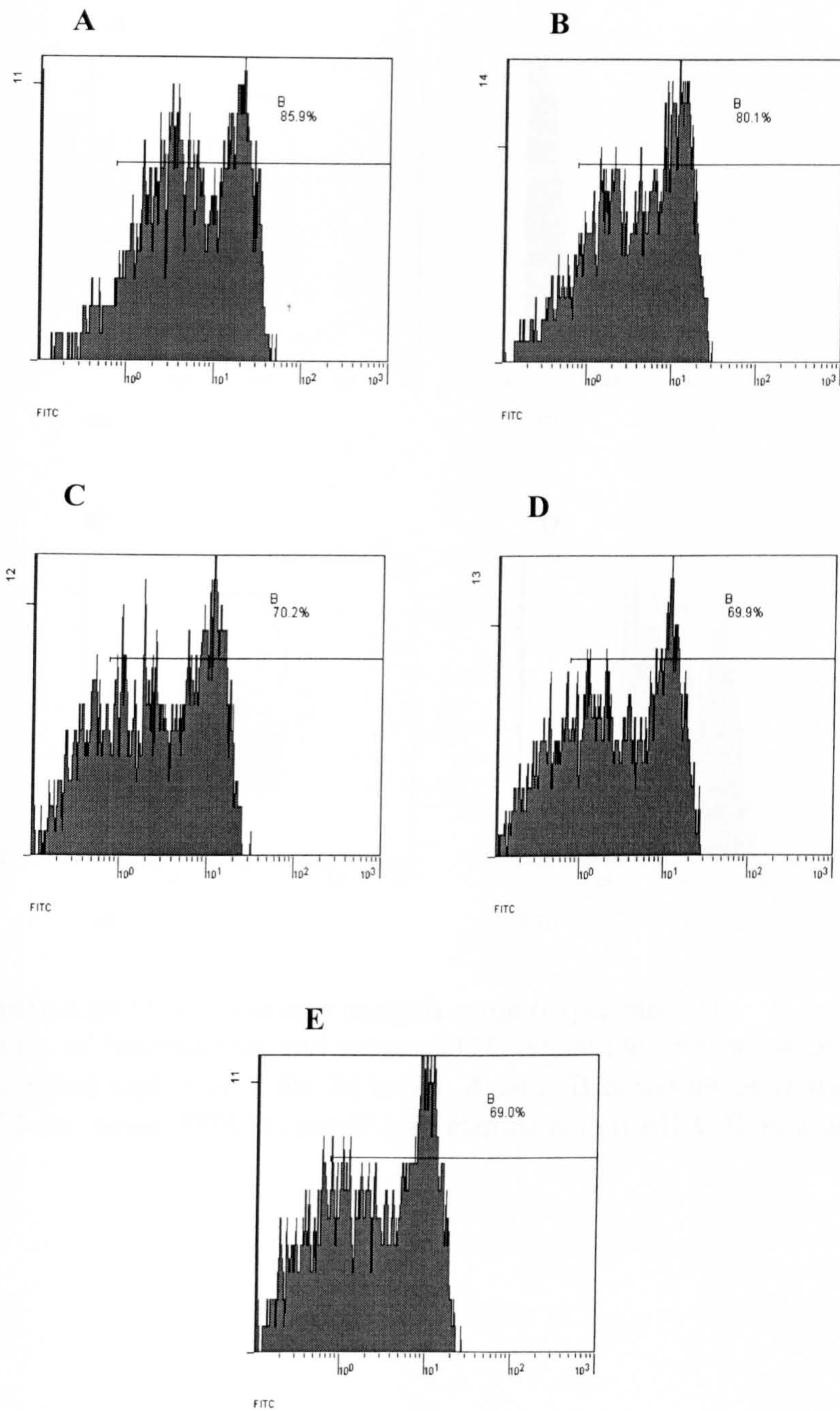


Figure 3.23- EqIFN- γ upregulation of MHC II (experiment 1) *. Enriched T cells were incubated for 24 hours with dilutions of bacIFN72 or parental virus preparations and stained with α -MHCII/ α -mouse:FITC. Figures A to D show increasing dilutions of IFN- γ (10^{-1} to 10^{-4} , respectively) and figure E corresponds to a 10^{-1} dilution of parental vector negative control.

*N.B.- Experiments were performed with blood from two individual animals with similar results.

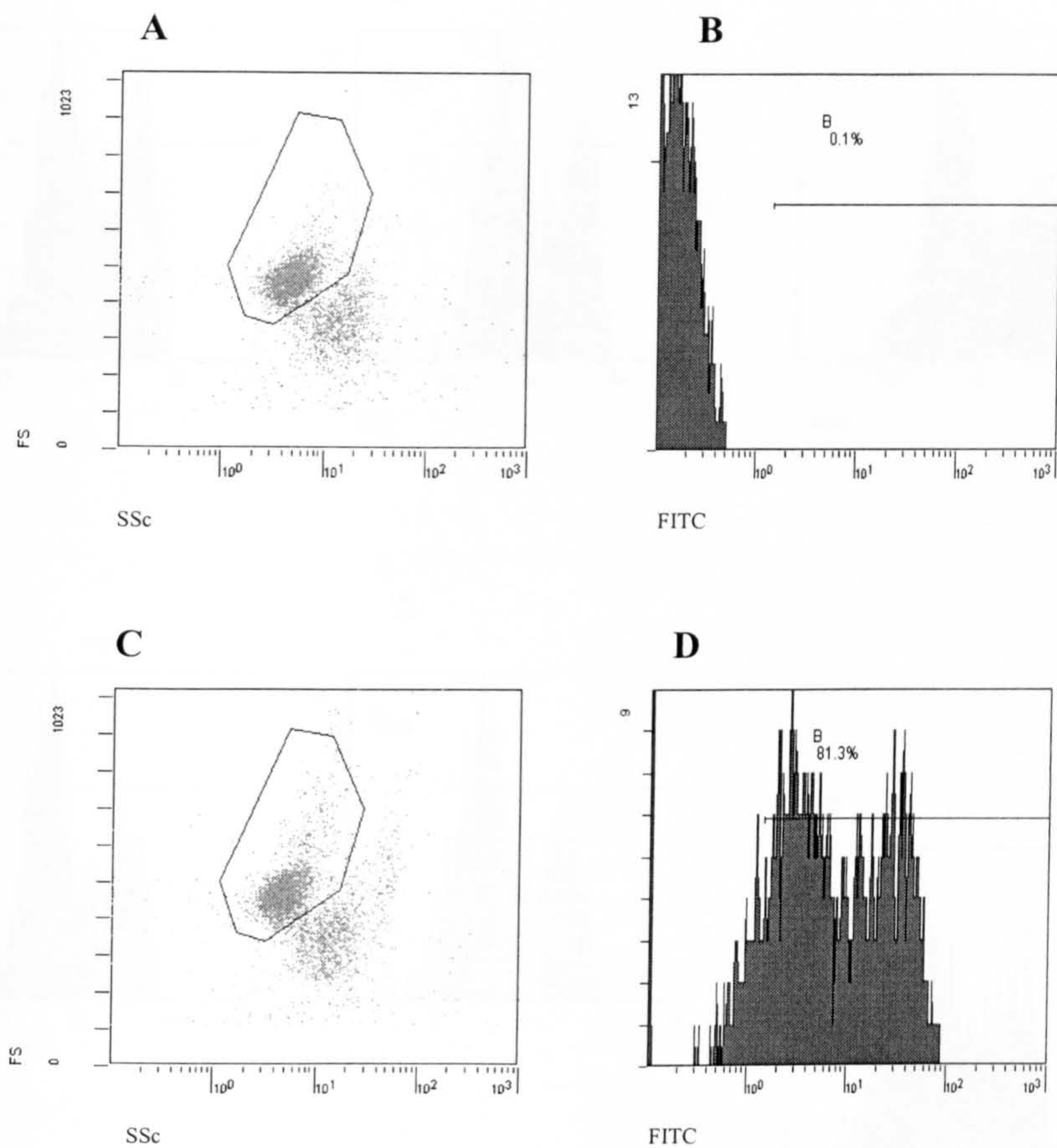


Figure 3.24 Flow cytometry analysis setup (experiment 2). Gating of lymphocytes and setting FITC acquisition interval as before for cells stimulated with rhIL-2 for 24 hours. A and B represent cells stained with α -gp24 / α - mouse:FITC; C and D cells stained with α -MHC II / α -mouse:FITC

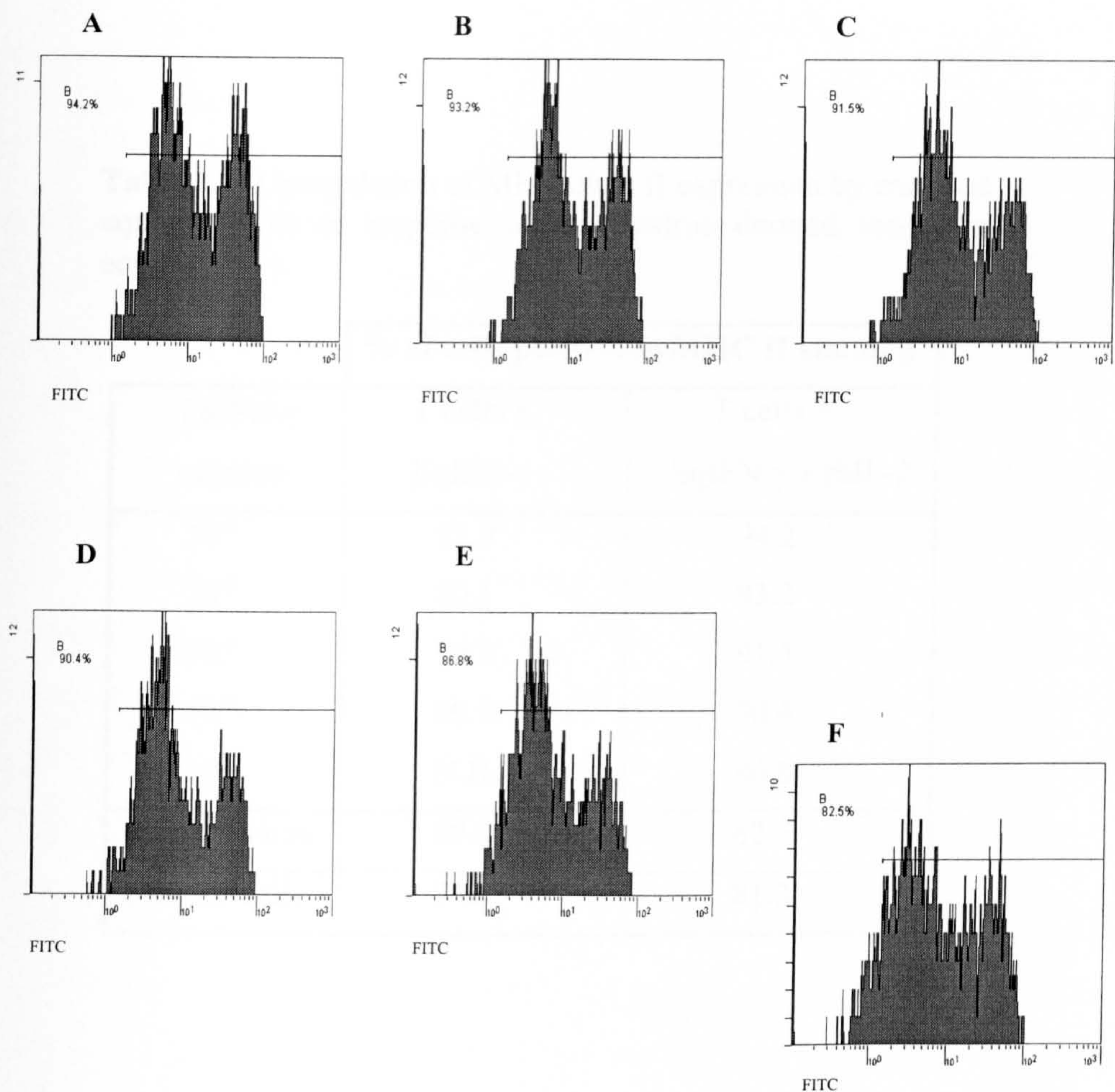


Figure 3.25 EqIFN- γ upregulation of MHC II (experiment 2) *.

Enriched T cells incubated 24 hours with rhIL-2 and dilutions of BacIFN72 or Bacwt control preparations, were stained with α -MHCII/ α -mouse:FITC. Figures A to E show increasing dilutions of IFN- γ (10^{-1} to 10^{-5} , respectively) and figure F corresponds to a 10^{-1} dilution of negative control.

* N.B.- Experiments were performed with blood from two individual animals with similar results.

Table 3.7 Upregulation of MHC class II expression by enriched equineT cells in response to baculovirus derived recombinant equine IFN- γ .

	% of cells positive to MHC II staining	
EqIFN- γ dilution	T cells + EqIFN- γ	T cells + EqIFN- γ + rhIL-2
10 ⁻¹	85.9	94.2
10 ⁻²	80.1	93.2
10 ⁻³	70.2	91.5
10 ⁻⁴	69.9	90.4
10 ⁻⁵	N.D.	86.8
Parental virus	69.0	82.5
Unstim. cells	70.8	81.3

3.4 Discussion

Equine IFN- γ was successfully produced in bacterial, baculovirus and mammalian expression systems. The identity of the expressed products was confirmed both by western-blot and by alternative methods (SDS-PAGE, PCR and northern blot analysis, respectively).

Antipeptide antibodies showed poor or no affinity for protein expressed in bacteria. This was possibly due to misrepresentation of the actual protein epitopes by the immunising peptides. Alternatively, folding of the mature protein conceals the specific epitopes represented by the peptides, making them unavailable for binding to the antibodies. Although the gels were run under denaturing conditions it is possible that this was not enough to completely unfold the protein.

The alternative strategy for antibody production resulted in the generation of a polyclonal antibody preparation able to strongly recognise not only its GSTeqIFN- γ immunogen, but also recombinant IFN- γ derived from baculovirus and mammalian expression systems. Additionally the antibody is cross reactive to human and feline species of IFN- γ .

Both baculovirus and mammalian expressed eqIFN- γ revealed a higher molecular weight, when compared with the GST derived protein control. This result is consistent with post-translational glycosylation of IFN- γ in eukaryotic expression systems. Still to prove, remains the usefulness of this polyclonal preparation in the detection of natural eqIFN- γ , as a tool for the dissection of immunological functions and pathology of the horse.

The low level of protection (640 to 2560 U/ml) exerted by eqIFN- γ against VSV in bovine kidney cells (MBDK), when compared to that reported for bovine IFN- γ , is possibly a consequence of the species specific character of IFN- γ activity, although cross reactivity between bovine and ovine species of IFN- γ as been documented (Martin *et al*, 1996 in Pastoret *et al*, 1998). Nevertheless, the bioactivity of

recombinant eqIFN- γ was confirmed by early experiments with the same assay system but using an equine cell line (EEK). In EEKs, baculovirus derived eqIFN- γ showed an activity corresponding to 10^5 laboratory units of IFN- γ per ml. This value matches those obtained by Zucker *et al*, (1993) for canine IFN- γ and of Argyle *et al* (1998) for the feline species. Unfortunately, we have lately been unable repeat these results. A plausible explanation would be the loss of sensitivity to of the cells (EEK) to eqIFN- γ , caused by increasing passage number. A similar observation was reported in the case of WISH cells in response to human IFN- γ (Lewis, 1987).

In the proliferation assay, response to eqIFN- γ alone was characterised by a mild incorporation of tritiated thymidine. However, when IFN- γ treated cells were spiked with mitogen (ConA), the proliferative response represented almost a tenfold increase. These results are consistent with observations that IFN- γ expands mitogen induced proliferation of lymphocytes (Landolfo *et al*, 1988). Another significant observation is that responses to both IFN- γ alone or IFN- γ plus ConA are characterised by linear, dose dependent, cell growth curves, which correlates with unequivocal proliferative activity of the samples tested. However these results need ideally to be confirmed since, due to time constraints, the assay was performed in only one batch of recombinant eqIFN- γ .

In the case of MHC class II regulation on enriched T cells, was observed a dose dependent variation in the percentage of cells expressing MHC II upon stimulation with eqIFN- γ . Nevertheless, the small interval between basal and maximal expression of MHC II and the non linearity of the increments make difficult the interpretation of the results obtained in absolute values. More importantly, though, when in co-stimulation with rhIL-2, the pattern of MHC upregulation by eqIFN- γ shifted from the dim to the bright population. As discussed before, resting T cells constitutively express low levels of MHC class II molecules, but can nevertheless be induced to increase the expression level. Activated T cells, on the other hand, not only are able to express larger amounts of MHC II but do it in a very specific and inducible way, possibly by reduction of the threshold for MHC II induction in response to antigens and particularly to IFN- γ . This fact is the basis of the association of increased

capacity in expressing MHC II, with equine T cell activation and development into an effector/memory state (Lanzavecchia, 1990; Altin and Sloan, 1997). Because IL-2 is a competent activator of T cells (originally named T cell growth factor) its plausible that, in the present system, IL-2 increased the susceptibility of T cells to IFN- γ activity.

Still, the heterogeneity of the cell population, precludes a definitive correlation between eqIFN- γ dose and the upregulation of MHC II molecules. This problem could be addressed by the use of an inducible cell line with negligible basal expression of MHC II. In such cell lines (e.g. B cell lymphoma) addition of IFN- γ leads to a major shift in the pattern of MHC II expression with an increase of mean fluorescence of up 100 fold. Unfortunately we were not able to secure such a cell line for the horse. This was in fact the reason for enrichment of the PBL population in T cells, in an attempt to increase cellular uniformity.

Another approach to the problem would be the simultaneous (double) labelling of MHC class II molecules and of lymphocyte subset markers (e.g. CD3 for T cells; CD19 for B cells). This enables the tracking of discrete populations of cells and the observation of small variations in MHC II expression by those cells. However, this technique requires antibodies with different fluorochromes (e.g. FITC, PE) and, more importantly, at least one of the antibodies to be directly conjugated with fluorochrome. There are no commercially available, directly conjugated antibodies against equine antigens, and this approach was not applied.

In view of the inconsistent and subtle biological activity demonstrated by the eqIFN- γ preparations tested, much work remains to be done not only in the optimisation of the assays but also, and perhaps more importantly, in the production of purer protein preparations. Significantly, the biological potency of the preparations does not correlate with the amount of recombinant protein detected by western blot. There are two main explanations for this observation. Firstly, that recombinant eqIFN- γ is being produced largely in an inactive form and in this case the preparations would probably benefit of more refined methods of protein purification methods (e.g. HPLC; FPLC).

Secondly, we could postulate that IFN- γ is not as active in the horse as in other species, which would in part be settled if sufficiently pure preparations of natural equine IFN- γ were obtained, that could be tested in the present assay systems. Nevertheless the second does not seem to apply in view of preliminary results obtained with the CPE reduction assay in EEKs and with the proliferation of T cells costimulated with mitogen.

Finally, attempts were also made, in the CPE reduction assay, to neutralise the biological activity observed by means of the existing polyclonal antibody, although without success (results not shown). This was probably due to the inability of the polyclonal to bind to the receptor binding domains of equine IFN- γ and therefore to specifically neutralise its activity. Steps are now being taken, both through collaborations and within the department, for the development of monoclonal antibodies against eqIFN- γ that will prove invaluable in the optimisation of the present assays.

CHAPTER 4

CLONING OF EQUINE IL-12 AND IL-18

4.1 Introduction

4.1.1 Interleukin-12 (IL-12)

4.1.1.1 Molecular structure and production of IL-12

Interleukin-12, the only heterodimeric cytokine isolated to date, was originally identified independently by two groups investigating the activity of a novel factor in lymphocytes and NK cells. The first group (Kobayashi *et al*, 1989) described a factor that could stimulate NK cell functions, NK cell stimulatory factor (NKSF). The second group (Stern *et al*, 1990) identified a molecule that activated cytolytic lymphocyte activities and was named accordingly, cytotoxic lymphocyte maturation factor (CLMF). It was only when both these factors were cloned (Wolf *et al*, 1991 and Gubler *et al*, 1991, respectively) that it became clear that this was in fact one molecule and the name IL-12 was proposed.

IL-12 is composed of two polypeptide chains, p35 and p40, covalently linked by a disulfide bond (figure 4.1). The p35 gene was mapped to human chromosome 3 (3p12-3q135.2) and to murine chromosome 3. The human p35 gene encodes a 253 amino acid polypeptide with two potential translation initiation codons (residues 1 and 35). Translation from the second methionine gives rise to a typical hydrophobic signal peptide (22 residues long) followed by a mature protein of 197 amino acids (with a predicted molecular weight of 27.5kDa) containing seven cysteine residues and three consensus N-linked glycosylation sites. Differential glycosylation leads to molecular heterogeneity of natural IL-12 p35, ranging from 30kDa to over 35kDa, as observed under denaturing conditions. Similarly, p40 is encoded by a gene mapped to human chromosome 5 (5q31-q33) and to murine chromosome 11. Human p40 is composed of eight exons and seven introns and encodes for an open reading frame of 328 amino acids in length that includes a N-terminal 22 amino acid long hydrophobic leader sequence. The mature protein, with a predicted molecular weight of 34.7 kDa, features ten cysteine residues, four possible N-linked glycosylation sites and a consensus heparin binding site. Natural IL-12 p40, has a molecular weight heterogeneity from 36 to over 40 kDa, under denaturing conditions, depending on the

extent of glycosylation (Wolf *et al*, 1991; Gubler *et al*, 1991, reviewed in Trinchieri, 1995).

The p40 and p35 subunits of the mouse IL-12 share, respectively, 70% and 60% amino acid sequence identity to their human counterparts.

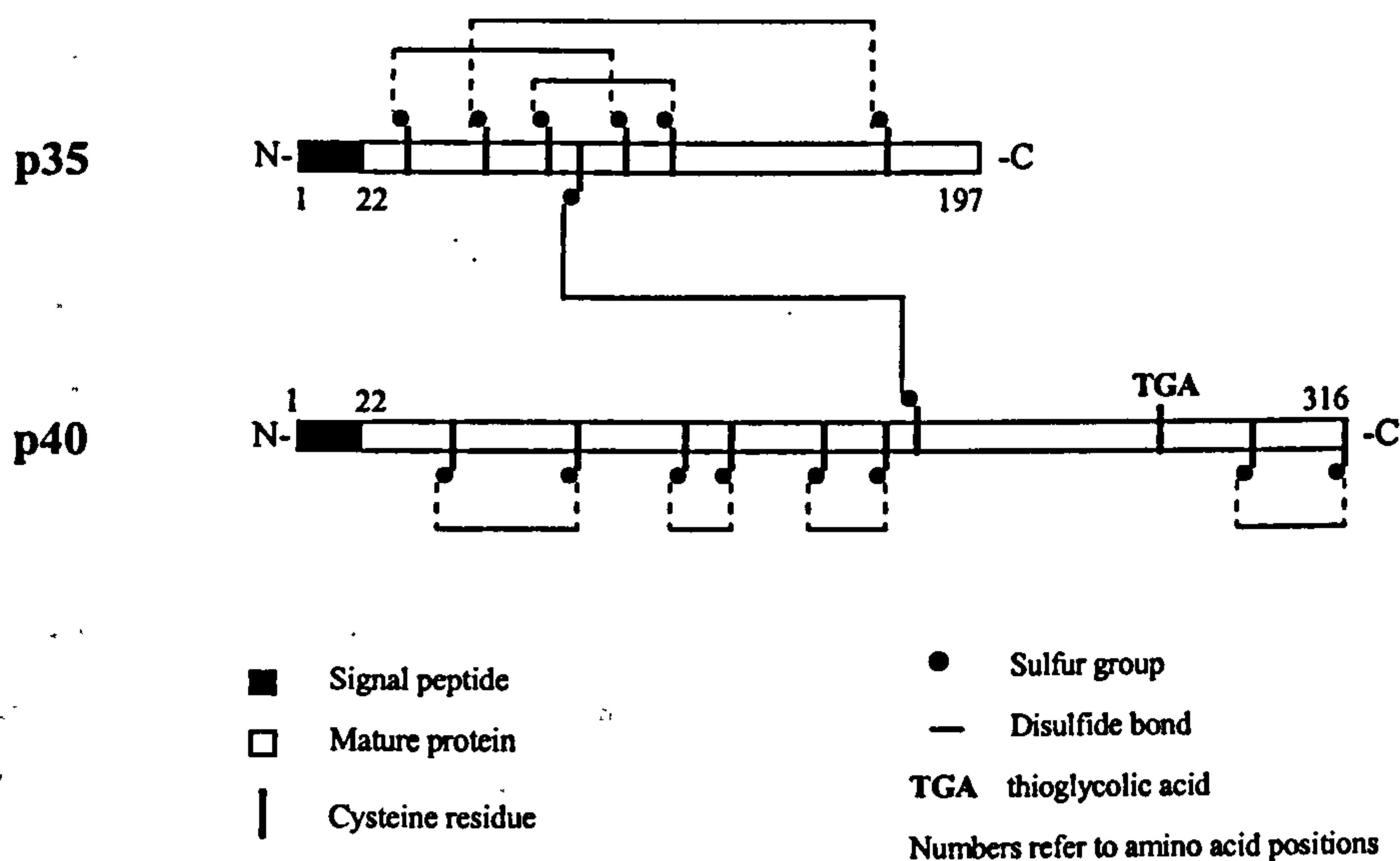


Figure 4.1 Structure of the human IL-12 heterodimer (Trinchieri, 1998). Showing intra and inter-chain disulfide bonds. Cys₇₄ of the p35 subunit and Cys₁₇₇ of p40 are involved in interchain covalent linking. All other cysteine residues in both molecules are involved in intra-chain bonds except for Cys₂₅₂ of p40 that is cysteinylated with thioglycolate (TGA) and therefore not available for disulphide bridging.

While there is no sequence homology between IL-12 p35 and p40 subunits, p35 shares significant homology with IL-6 and G-CSF and has, similarly to several other cytokines, an α -helix-rich structure (Merberg *et al*, 1992). On the other hand, p40 is not homologous to other cytokines, but belongs to the hemopoietin receptor family, resembling the extracellular domain of IL-6 receptor (Gearing *et al*, 1991). It is therefore possible that a primordial cytokine (the p35 equivalent) by binding to its soluble receptor (the p40 equivalent) gave rise to the heterodimeric IL-12 during evolution. This notion is supported by observations that other cytokines (e.g. IL-6, IL-

11) can bind their soluble receptors without compromising cellular receptor binding and signal transduction (Trinchieri, 1998).

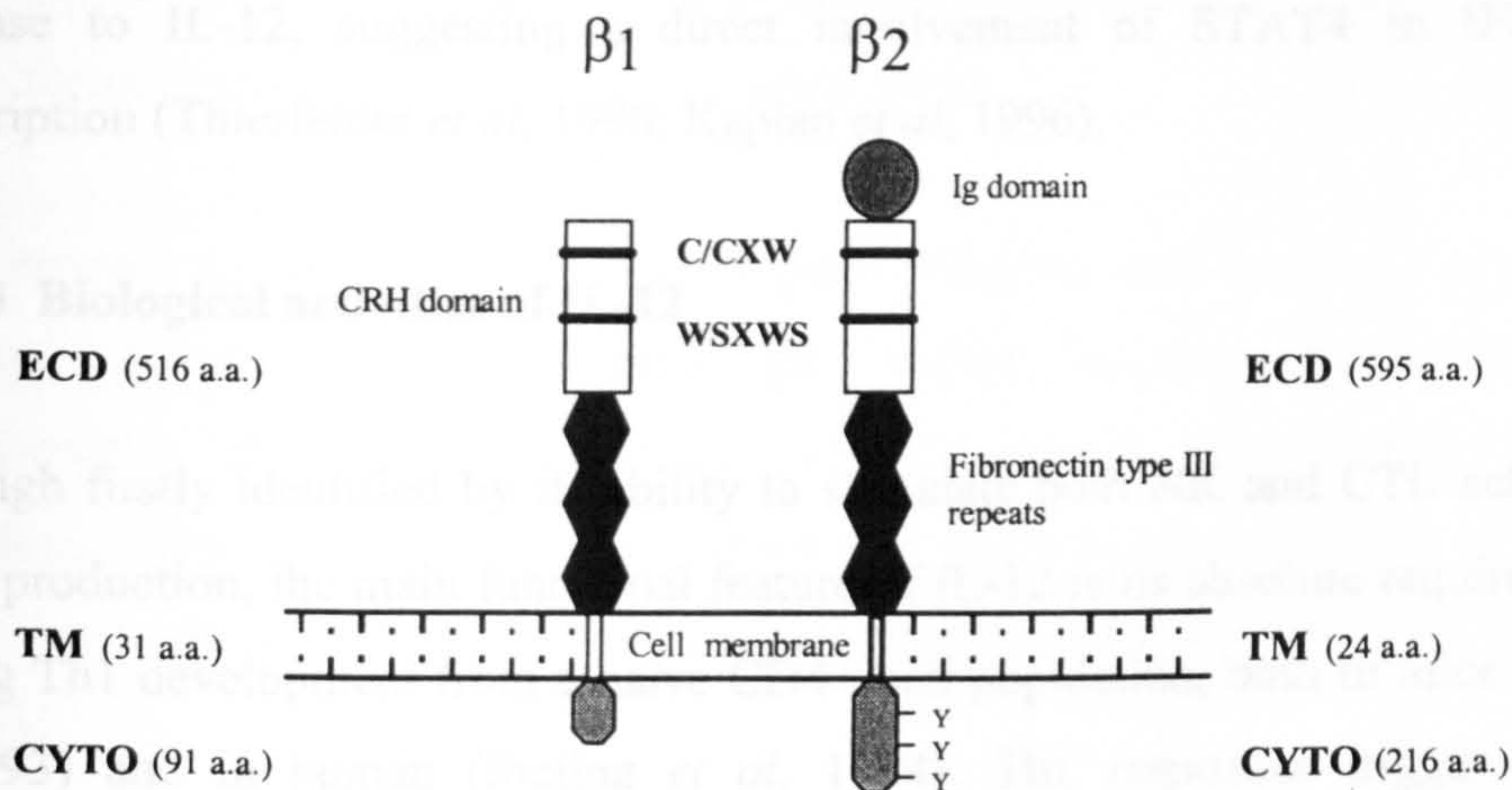
Although originally identified in supernatants of B cell lines transformed with Epstein-Barr virus (Kobayashi *et al*, 1989), it is now known that the main sources of IL-12, *in vivo*, are activated macrophages and dendritic cells, mainly in response to intracellular parasites, bacteria and their products (reviewed in Trinchieri, 1995). Other cellular producers of IL-12 include neutrophils, Langerhans cells, keratinocytes, microglia and astrocytes (reviewed in Storkus *et al*, 1998).

The production of IL-12 is complex, as simultaneous expression of p35 and p40 is required for secretion of bioactive heterodimer. Although most somatic cells constitutively express low levels of p35 transcripts, p35 protein has not been detected, suggesting that this chain is efficiently secreted only when associated with p40. Furthermore, the expression of p40 is highly regulated and restricted to cells able to produce bioactive IL-12. It has been reported that stimulated macrophages produce a large excess of p40 relatively to IL-12 heterodimer, and that p40 possesses an antagonistic effect over IL-12. Also p40 homodimer was found to bind IL-12 receptor without mediating biological responses. Taken together these observations suggest that p40 expression plays an important regulatory role in IL-12 production and activity (reviewed in Stern *et al*, 1997; Trinchieri, 1998).

4.1.1.2 IL-12 receptor and signalling

IL-12 receptor (IL-12R) is primarily expressed on activated T and NK cells (Desai *et al*, 1992). It is composed of two subunits designated IL-12R β 1 (of 100kDa) and β 2 (of 130kDa). Both subunits are type I transmembrane glycoproteins and belong to the gp130 subgroup of the cytokine receptor superfamily (figure 4.2). Radiolabelled ligand and expression studies revealed the presence of both high and low affinity IL-12 binding sites in activated lymphoblasts. These studies also suggest that the β 1 subunit provides most of the binding energy, whereas the β 2 subunit is the primary signal transducing element (reviewed in Presky *et al*, 1996). Control of IL-12R β 2

expression constitutes a critical mechanism for regulating IL-12 responsiveness. This is well illustrated by the increased response to IL-12 of developing TH1 cells through IFN γ driven upregulation of IL-12R β 2 and the loss of response to IL-12 observed in TH2 cells mediated by IL-4, IL-10 and TGF- β downregulation of the β 2 receptor subunit (Gollob *et al*, 1997).



ECD - extracellular domain; **TM** - transmembrane domain; **CYTO** - cytoplasmic domain

Figure 4.2 Structure of the human IL-12 receptor (modified from Stern *et al*, 1997; Trinchieri, 1998). Functionally important residues include the characteristic cytokine receptor superfamily motifs (C/CXW and WSXWS) within the cytokine receptor homology (CRH) domain, responsible for ligand binding, and the β 2 chain cytoplasmic tyrosine residues (Y) involved in signal transduction.

The transduction of signal by IL-12 is still not completely understood. Initial reports described IL-12 induced phosphorylation of mitogen activated protein kinase (MAPK) in T cells (Pignata *et al*, 1994) and of p56LCK in NK cells (Pignata *et al*, 1995). Nevertheless, it is now thought that the JAK/STAT pathway is the main mechanism involved in mediating IL-12 biological activities. This assumption is supported by evidence that treatment of T and NK cells with IL-12 induces rapid phosphorylation of both JAK2 and Tyk2 kinases followed by activation of members of the STAT family of transcription factors (Yu *et al*, 1996). Three components of the STAT family (STAT1, STAT3 and STAT4) are phosphorylated and associate as homo or heterodimers in response to IL-12 before translocation to the nucleus and activation of gene transcription. The relative importance of each of the STAT proteins

has not yet been established although STAT4 has so far only been found to be activated in response to IL-12 and IFN α , contrary to the involvement of STAT1 and STAT3 in the activity of many cytokines (Cho *et al*, 1996). The importance of STAT4 in IL-12 signalling was further emphasised when STAT4 knockout mice were observed to have a phenotype equivalent to that of IL-12 or IL-12R knockout mice. Additionally T and NK cells from STAT4 knockouts do not produce IFN- γ in response to IL-12, suggesting a direct involvement of STAT4 in IFN- γ gene transcription (Thierfelder *et al*, 1996; Kaplan *et al*, 1996).

4.1.1.3 Biological activities of IL-12

Although firstly identified by its ability to stimulate both NK and CTL activity and IFN- γ production, the main functional feature of IL-12 is its absolute requirement for driving Th1 development from a naïve CD4 $^{+}$ cell population, both in mice (Hsieh *et al*, 1993) and in human (Sieling *et al*, 1994). Th1 responses trigger profound modulatory effects on both cellular and humoral immunity that are largely mediated by IFN- γ (discussed in chapter 1).

Other activities of IL-12 (reviewed in Ma *et al*, 1997; Gately *et al*, 1998; Storkus *et al*, 1998; Trinchieri, 1998) include:

- production of other cytokines, including TNF- α , GM-CSF, IL-8, IL-3 from T and NK cells when these cells are co-induced by other stimuli like anti-CD3, anti-CD16, anti-CD28 or phorbol diesters. Inhibitory effect over the production of IL-4 and IL-10;
- enhanced proliferation of NK and T cells upon pre-activation (by antigen) or costimulation. In T cells the proliferation effect of IL-12 is observed on both CD4 $^{+}$ and CD8 $^{+}$ and is not mediated by endogenous IL-2. In this activity IL-12 synergises with IL-18 and IFN- γ and is antagonised mainly by IL-4, IL-10 and TGF- β (by downregulation of the IL-12R);
- synergism with IL-2 in the generation of lymphokine-activated killer (LAK) cell activity against sensitive target cells (e.g. tumour cells, virus infected cells). LAK cells are cytotoxic cells comprising NK cells and non-MHC restricted CTLs. The ability of IL-12 to enhance cytotoxic activity is due both

to the induction of expression of granule associated proteins (e.g. perforin, granzyme) and to the direct cell proliferation activity of IL-12 that leads to expansion of cytolytic effector cells;

-IL-12 is chemotactic for human NK cells in vitro and stimulates their interaction with vascular endothelium via the LFA-1/ICAM-1 pathway;

-synergises with stem cell factor in the differentiation of lymphohematopoietic precursors and with IL-3 in the production of granulocyte macrophage colony forming unit from hematopoietic progenitors.

IL-12 appears to have a direct role in preventing the occurrence of spontaneous tumours. Indeed, tumour transplantation studies in mice have shown that administration of IL-12 dramatically reduced tumour growth and metastasis formation. These studies have also shown that the anti-tumour effect of IL-12 is mainly due to the stimulation of CTL activity and independent of NK cells (Brunda *et al*, 1993).

A variety of studies employing murine models of infection have confirmed an important role for IL-12 in the resistance to a variety of intracellular parasites including bacteria, fungi, protozoans and viruses, mediated, in part, if not totally by IFN- γ (Gately *et al*, 1998). The requirement for IL-12 appears to be an early event in infection as mice infected with *Toxoplasma gondii* succumb to infection if treated with anti-IL-12 antibodies during the acute phase of infection but not if the treatment is applied when the process has evolved to chronicity (Gazzinelli *et al*, 1992). The importance of IFN- γ production in response to IL-12 was highlighted in experimental viral infection studies. It was found that mice receiving inactivated pseudorabies virus in conjunction with IL-12 were resistant to lethal virus challenge whereas IFN- γ R knock-out mice succumbed to challenge (Schijns *et al*, 1995). Likewise, the protection conferred by IL-12 to infection with murine cytomegalovirus was abrogated by using anti-IFN- γ antibodies or depletion of NK cells (Orange *et al*, 1995).

In conclusion, by being rapidly produced in response to infection, IL-12 contributes to an early innate response by activation of NK cells which, through increased production of IFN- γ , mediate activation of macrophages and inflammatory responses. At the same time it sets the stage for an antigen-specific cell mediated response by skewing Th and CTLs to type 1 (Th1 and TC1, respectively) effector responses complemented by the development of an appropriate immunological memory (Gately *et al*, 1998; Trinchieri, 1998).

4.1.2 Interleukin-18 (IL-18)

4.1.2.1 Molecular structure and production of IL-18

IL-18 was first identified in the serum (Nakamura *et al*, 1989) and purified from liver extracts of mice (Nakamura *et al*, 1993), primed with *Propionibacterium acnes* and challenged with LPS, as a factor involved in the onset of endotoxic shock through the induction of IFN- γ production. Consequently the name interferon-gamma-inducing factor (IGIF) was proposed (Okamura¹ *et al*, 1995). Primers designed from the purified protein were used to probe liver cDNA libraries of this murine model, leading to the isolation and cloning of a full length cDNA product (Okamura² *et al*, 1995). Subsequently, the murine probes were also used clone the human equivalent from the erythroleukemic cell line K 562 (Ushio *et al*, 1996). The cloned murine and human IL-18 encode precursor polypeptides of 192 and 193 a.a., respectively, with an apparent molecular weight of 24kDa as assessed by electrophoresis under denaturing conditions. Human and murine IL-18 have 65% homology at the protein level. Features of the protein sequence (figure 4.3) include an unusual non-hydrophobic signal peptide sequence, the absence of N-glycosylation sites, the (F-X₍₁₂₎-F-X-S-X₍₆₎-F-L) IL-1 signature-like sequence (reviewed in Kohno and Kurimoto, 1998) and an overall β -trefoil structure, comprising 12 β -sheets, resembling that of the IL-1 family of proteins (Bazan *et al* 1996).

IL-18 is produced as an inactive precursor polypeptide (proIL-18) that requires cleavage, at the Asp-X processing site, by the extracellular protease interleukin-1 β -converting enzyme (ICE), for the generation of the biologically active mature IL-18

with 18kDa (figure 4.3). The importance of ICE in the generation of bioactive IL-18 was elucidated when mice deficient in ICE were found to have circulating levels of IFN- γ matching those detected in IL-18 “knock-out” mice, under the same stimulation conditions (Ghayur *et al*, 1997). ICE belongs to the caspase family of proteins and another member of this family (caspase-3) was found to cleave IL-18, although generating biologically inactive fragments (reviewed in Fantuzzi and Dinarello, 1999).

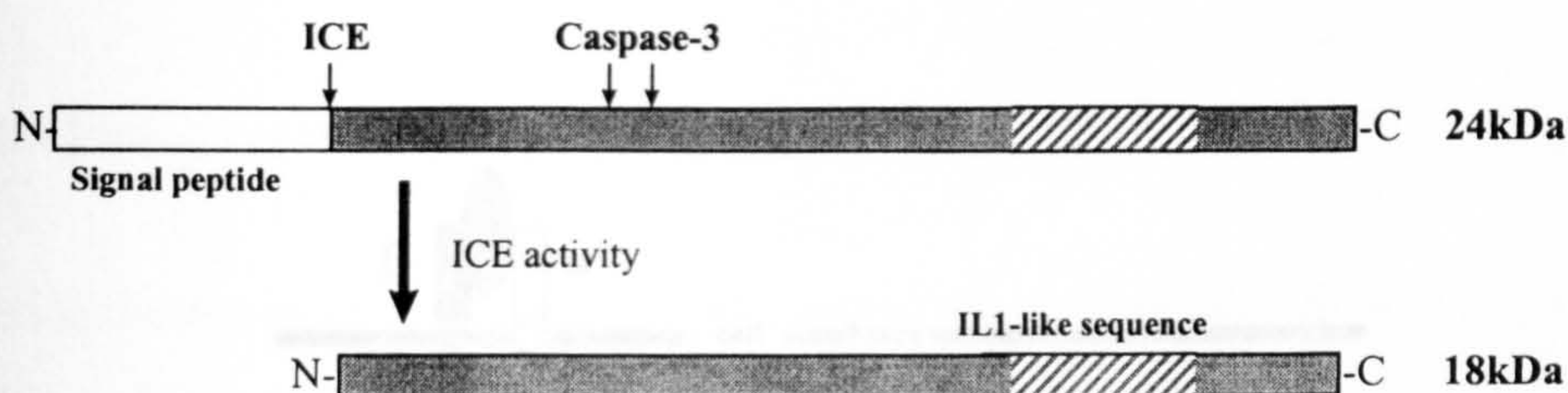


Figure 4.3 Polypeptide structure and processing of IL-18 (modified from Okamura *et al*, 1998; Fantuzzi and Dinarello, 1999).

IL-18 is expressed primarily by monocytes/macrophages, but also by other cell types including keratinocytes (Stoll *et al*, 1997), osteoblasts (Udagawa *et al*, 1997) and epithelial cells of the intestine (Takeuchi *et al*, 1997) and adrenal cortex (Conti *et al*, 1997). Nevertheless, due to its requirement for post-translational processing, the detection of IL-18 RNA transcripts does not mean that the cells produce active IL-18. In fact, although RNA message can be readily detected in unstimulated liver macrophages (Kupffer cells), only after LPS stimulation do these cells produce bioactive IL-18 (Okamura *et al*, 1998).

4.1.2.2 IL-18 receptor and signalling

The IL-18 receptor (IL-18R) complex appears to be formed, like that of IL-1, by an α chain (IL-18R α), responsible for binding and a β -chain (IL-18R β) which is mainly involved in signalling. The IL-18R α was found to be the molecule previously known

as IL-1R related protein (Torigoe *et al*, 1997) but so far without a ligand as it did not bind any of the IL-1 family of proteins (Parnet *et al*, 1996). IL-18R β is structurally and functionally similar to the IL-1 R accessory protein (IL-1R-AcP) having initially been named accessory protein like (AcPL) (Born *et al*, 1998). Cells can express both low affinity receptors formed by IL-R α alone and high affinity receptors that are complexes of the two chains. A third protein with IL-18 binding activity (IL-18BP) has recently been isolated from human urine and is thought to be the soluble form of the extracellular portion of IL-18R α , serving as a decoy receptor for IL-18 (Novick *et al*, 1999).

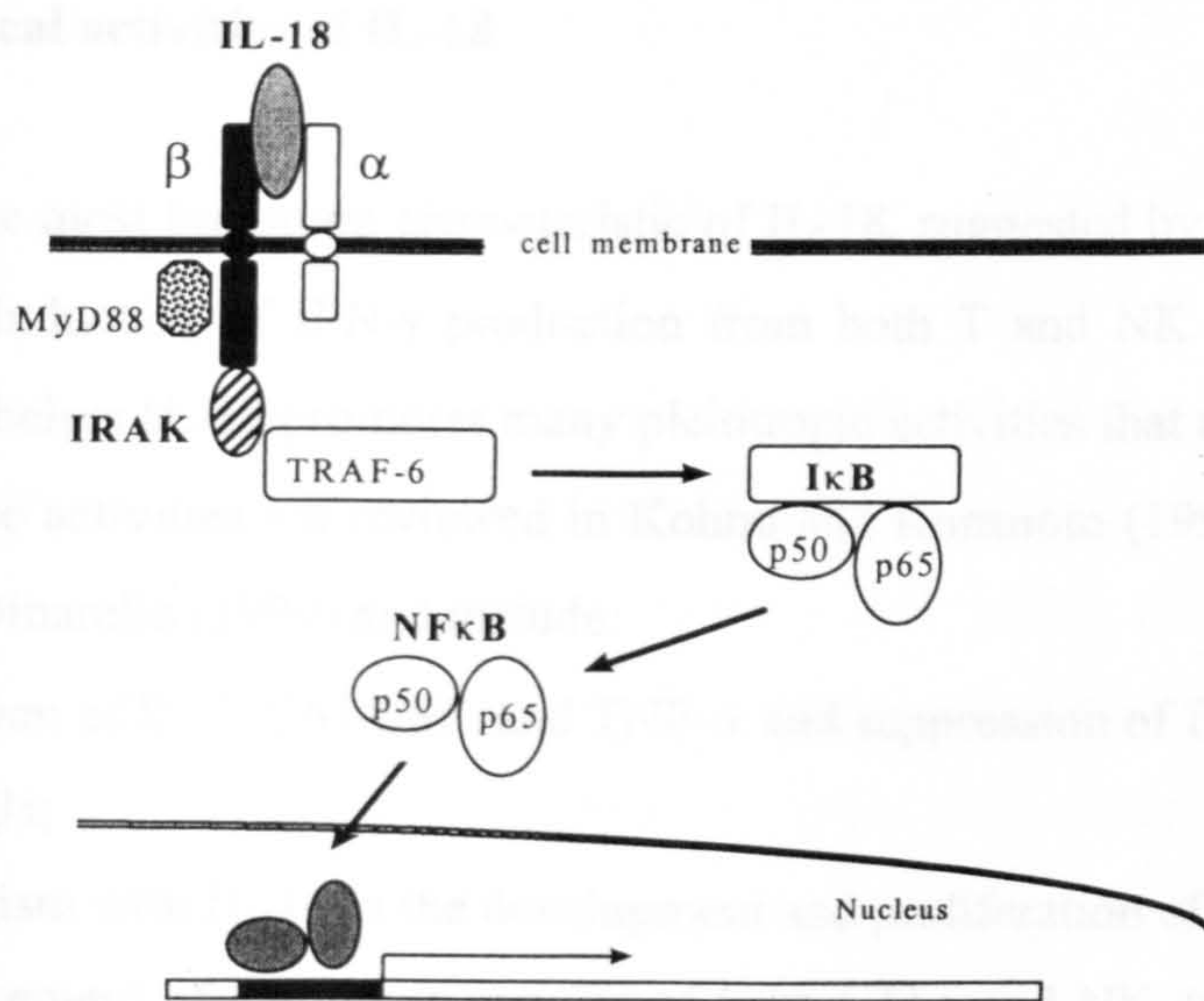


Figure 4.4 Signal transduction by IL-18 (modified from Fantuzzi and Dinarello, 1999).

Following IL-18 binding to its receptor, oligomerisation of the receptor complex initiates the signalling cascade responsible for the transcription of responsive genes (figure 4.4). The intracellular domain of receptor β -chain phosphorylates the IL-1 receptor associated kinase (IRAK) possibly with the mediation of an adapter molecule named MyD88, that allows docking of IRAK to the receptor. Once phosphorylated IRAK dissociates from the receptor complex and phosphorylates the TNF receptor associated factor-6 (TRAF-6). The nuclear factor- κ B (NF κ B), in its resting form, is bound to an inhibitory protein named I κ B. Phosphorylation of I κ B by TRAF-6 leads

to its degradation by ubiquitin with release of the p50 and p65 components of NF κ B. Activated NF κ B translocates to the nucleus activating gene transcription (Reviewed in May and Ghosh, 1998; Fantuzzi and Dinarello, 1999). In addition, p56LCK and p42MAPK were also found to be activated and associate following IL-18 induction, but their function as signalling components of IL-18 is still not understood (Tsuji-Takayama, *et al*, 1997). Also obscure is the process by which IL-18 binding to its receptor leads to activation of AP-1, found to be the major DNA binding protein associating with the IFN- γ gene promoter following IL-18 stimulation of T cells (Barbulescu *et al*, 1998).

4.1.2.3 Biological activities of IL-18

Functionally the most important characteristic of IL-18, suggested by its former name (IGIF), is the induction of IFN- γ production from both T and NK cells (described below). Nevertheless IL-18 promotes many pleiotropic activities that are not mediated by IFN- γ . These activities are reviewed in Kohno and Kurimoto (1998), Okamura *et al* (1998) and Dinarello (1999) and include:

- induction of IL-2, GM-CSF, and TNF- α and suppression of IL-10 production by T cells;
- synergism with IL-12 in the development and proliferation of Th1 cells;
- enhancement of cytotoxic activity of both CTLs and NK cells not only by the increased release of cytolytic substances (e.g. perforin, granzyme A) but also by increasing Fas-ligand expression leading to Fas mediated apoptosis of target cells;
- induction of chemokine production (e.g. IL-8) by monocytes and NK cells in synergism with TNF- α ;
- enhancement nitric oxide production and that of proinflammatory cytokines (e.g. IL-6, IL-1 β) by macrophages;
- role in bone remodelling by suppression of osteoclast differentiation mediated by GM-CSF.

IL-18 promotes increased resistance to viral infection through the enhancement of cytotoxic activity. Nevertheless, addition of IL-18 to human lymphoma cells (U937) infected with HIV-1, was found to increase production of virus linked to an increase in nitric oxide synthase activity (Okamura *et al*, 1998). IL-18, administered to mice, had a significant antitumour effect and induced specific immunity to the tumour. This effect was found to be promoted by the activation of NK cells independently but in synergism with IL-12 and IFN- γ (Osaki *et al*, 1998). The role of IL-18 in bacterial infection is controversial appearing to be dependent on the infection model used. In *Salmonella* infection the protection afforded by IL-18 administration is mediated by IFN- γ , whereas in the case of *Yersinia* infection, IL-18 promotes resistance independently of IFN- γ (Dinarello, 1999).

4.1.3 Induction of IFN- γ by IL-12 and IL-18

The single most important activity of both IL-12 and IL-18 is their potent synergistic induction of IFN- γ production primarily from T and NK cells but also from B cells (Yoshimoto *et al*, 1997) and macrophages (Munder *et al*, 1998). Results obtained *in vitro*, with various cell types revealed that, in every case, the combination of IL-12 and IL-18 was far more effective in inducing IFN- γ than either cytokine alone. Furthermore, the synergism is observed even with saturating concentrations of both molecules (Kohno and Kurimoto, 1998). This suggests that they act through different signalling pathways, leading to transcription through activation of different enhancer elements of the gene promoter with a rapid accumulation of IFN- γ mRNA. Indeed, in freshly isolated human T cells, IL-18 was reported to cause high IFN- γ gene promoter activity via its AP-1 site, whereas binding of STAT 4 to the IFN- γ promoter following IL-12 stimulation resulted in low level of gene transcription. However when IL-12 was added in combination with anti-CD3 and anti-CD28 antibodies (therefore mimicking full T cell activation) both STAT 4 and AP-1 sites of the IFN- γ promoter were activated, resulting in high level of transcription (Barbulescu *et al*, 1998). A further explanation for the synergism between this two cytokines is offered by the observation that IL-12 is able to directly upregulate the expression of IL-18R (Ahn *et al* 1997) while IL-18, at least indirectly through IFN- γ production, is able to have the

same effect over the expression of IL-12R β -chain (Dinarello, 1999). As yet unpublished data suggests that IL-18 is also able to directly upregulate expression of IL-12R (A. O'Garra personal communication).

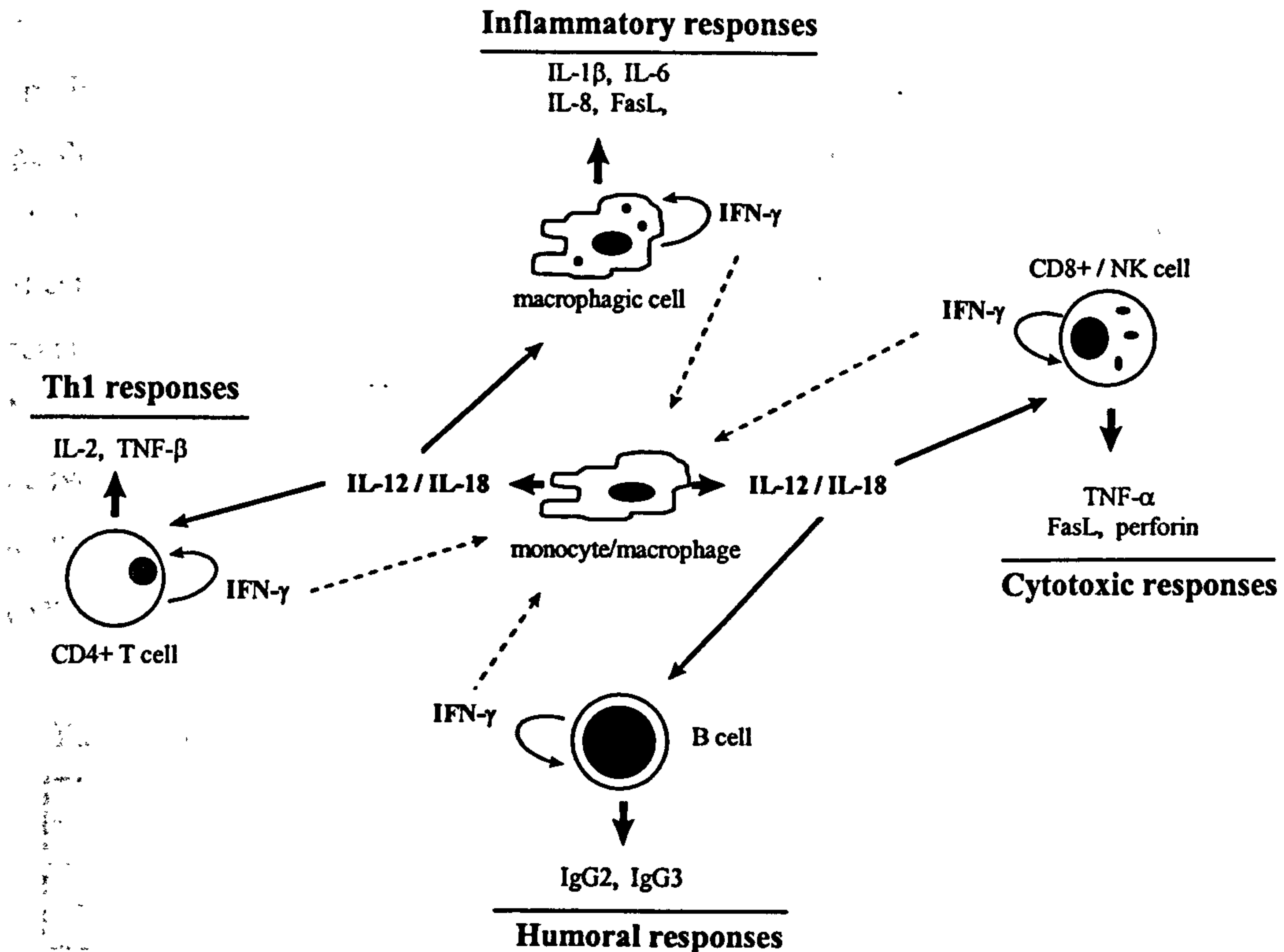


Figure 4.5 Integration of IL-12, IL-18 and IFN- γ activities in a Th1 context. Showing positive feed-back loops in the production of IFN- γ (solid lines) and IL-12/IL-18 (dashed lines). (modified from Dinarello, 1999).

The reciprocal receptor transmodulation, in addition to the fact that IL-12 and IL-18 have essentially the same cellular targets, is reflected in their cooperation in a significant number of activities that bridge innate and antigen-specific immune responses as well as inflammatory responses and that have IFN- γ production as a common denominator (figure 4.5). These include: optimal development of a CD4+ Th1 phenotype; proliferation and enhancement of cytolytic activity of T and NK cells; activation of macrophages and induction of activated B cells to shift antibody

production from neutralising (IgE and IgG1) to cytotoxic and complement-fixing (IgG2a/b and IgG3) subclasses in mice (Trinchieri, 1998; Dinarello, 1999).

The potent induction of IFN- γ production, associated with the other synergistical activities of IL-12 and IL-18 in host protection against intracellular pathogens, motivated us to isolate these cytokines in the horse. At the time the only sequences published for IL-12 were those corresponding to human (Wolf *et al*, 1991; Gubler *et al*, 1991), murine (Schoenhaut *et al*, 1992) and bovine (Zarlenga *et al*, 1995) species. Despite the level of homology these molecules have (table 4.1), cross reactivity studies of IL-12 between mouse and human showed that although mouse IL-12 has some biological activity over human lymphocytes, human IL-12 is not active in murine cells (Trinchieri, 1998). Furthermore, recombinant human IL-12 administered to non-human primates, gives rise to anti-human IL-12 antibodies (Sarmiento *et al*, 1994).

Table 4.1 Cross-species homology of IL-12 p40 and p35 peptides.

IL-12 p40	Human p40	Murine p40	Bovine p40
Human p40	-	66.7%	84.2%
Murine p40	66.7%	-	65.8%
Bovine p40	84.2%	65.8%	-
IL-12 p35	Human p35	Murine p35	Bovine p35
Human p35	-	58.9%	81.5%
Murine p35	58.9%	-	57.9%
Bovine p35	81.5%	57.9%	-

In the case of IL-18, the only two sequences available were the human (Ushio *et al*, 1996) and the murine (Okamura² *et al*, 1995) with an homology of 65% at the protein level. Again, although some bioactivity was detected by using murine recombinant protein in human cells this was 100-fold less active than recombinant human IL-18 under the same conditions (Konishi *et al*, 1997), highlighting once more the requirement for species-specific cytokines.

Previous experience obtained by the cloning of equine TGF- β (Penha-Goncalves *et al*, 1997-Appendix I) indicated that the cross-species homology of both IL-12 and IL-18 was sufficient to derive oligonucleotides from conserved regions of the molecules.

This would then enable the amplification of transcribed equine mRNA molecules, by RT-PCR, from suitable equine cells and under appropriate stimulation conditions.

Below is a description of the methods used in the amplification of equine IL-12 (eqIL-12) and IL-18 (eqIL-18) coding sequences as well as their cloning in plasmids to be co-administered in a DNA vaccination trial against equine influenza virus (chapter 6). The collaboration of the following co-workers in this part of the project is acknowledged: Ms J.L. Keanie in the cloning of IL-12 and IL-18; Miss N. Logan in the generation of full length IL-12 p35 and p40 cDNAs; Mrs L. McMonagle and summer students Claire Brown and Spencer French in the generation of expression constructs and Mrs Dorothy Reid in the preliminary expression of IL-12 and IL-18. Most of the results of the present chapter were submitted for publication (Nicolson *et al*, 1999 – Appendix I).

4.2 Materials and methods

Because the main producers of IL-12 and IL-18 are macrophagic cells, isolation of eqIL-12 and eqIL-18 was attempted from primary cell cultures enriched in alveolar macrophages, derived from lung washes.

4.2.1 Preparation of enriched alveolar macrophages

Lung washes were performed in the post-mortem room of the veterinary school, Glasgow. After euthanasia and careful removal of lungs from horses, the lungs were cleaned from excess blood and a sterile plastic funnel was introduced in the trachea. The lungs were then filled, with up to one litre of ice cold Hank's Buffered Saline (HBSS), and gently massaged. Liquid was decanted directly to centrifuge bottles avoiding blood contamination. The lung wash was repeated twice more for a total of three times. Resulting wash fluid was pooled in centrifuge bottles, transferred to the laboratory in crushed ice and centrifuged at 1800g (4500 rpm in a J14 rotor) for 10 min at 4°C. After centrifugation the froth was removed, the supernatant discarded and the cells resuspended in HBSS. Cells were pooled and washed once more in HBSS by centrifugation as before. The supernatant was discarded and the cells were washed twice in RPMI 1640 with 10% FCS, resuspended in 5 to 10 ml of RPMI and counted by Trypan blue exclusion. Cells were diluted to 2×10^7 /ml in complete medium (RPMI 1640 with 5% FCS, 100 IU/ml of penicillin/streptomycin, 10 mM Hepes, 2 mM Glutamine and 5×10^{-5} M β -mercaptoethanol), and distributed into 162 cm² flasks (50 ml per flask with 10^8 cells), followed by overnight incubation at 37°C with 5% CO₂. The supernatant was removed and adherent cells were washed two times with complete medium. 40 ml of complete medium containing LPS at 10 µg/ml was added to each flask followed by incubation for 6 hours as before. The supernatant was removed and using a cell scraper the cells were detached, resuspended in 50 ml of complete medium and pelleted at 1200 rpm for 5 min in a bench top centrifuge. The pellet resultant from each flask was resuspended in 50 ml of complete medium and divided in two tubes and the cells centrifuged as before. The supernatant was discarded and

the pellets were snap-frozen in dry-ice with 100% ethanol. Frozen pellets were stored at -70°C until ready to perform mRNA extraction.

4.2.2 Amplification of equine IL-12 and IL-18 cDNAs

Preparation of cDNA

All the reagents used in the preparation of mRNA and cDNA were prepared in DEPC treated dH₂O (mat&meth) and all tubes and tips were RNase free.

Extraction of mRNA from stimulated equine “macrophages” was performed using the Quick Prep mRNA Isolation Kit (Pharmacia) observing the manufacturers instructions. Resulting mRNA yield and purity was assessed by spectrophotometry and treated with methylmercury hydroxide as follows. Up to 1 µg of mRNA was diluted in DEPC treated dH₂O to a volume of 20 µl and incubated at 65°C for 10 min and transferred to crushed ice. Methyl mercury hydroxide (2 µl of a 100mM solution) was added to the tubes and incubated at room temperature for 1 min. β-mercaptoethanol (4 µl of a 700mM solution) was added and the tubes incubated at room temperature for a further 5 min. The mRNA was reverse transcribed using the First Strand cDNA Synthesis Kit (Pharmacia) with an oligo-dT as primer, following the manufacturers instructions. The cDNA synthesis reaction was incubated at 37°C for 1 hour and the reaction was stopped by denaturation of the enzyme at 94°C for 5 min, and frozen at -70°C when not used immediately.

Primer design

Primers for PCR (table 4.2) were designed by aligning published nucleotide sequences of IL-12 and IL-18 from other species (figure 4.6). For IL-12 the sequences of human (Wolf *et al*, 1991; Gubler *et al*, 1991), murine (Schoenhaut *et al*, 1992) and bovine (Zarlenga *et al*, 1995) species were used. For IL-18 were used the human (Ushio *et al*, 1996) and murine (Okamura² *et al*, 1995) sequences.

P40

	-16	21	
Primer 1	TCCTGCCCAGAGCAAGATGTGTCAACCAGCAGTTGGTC		Human
	ATGCACCCTCAGCAGTTGGTC		Bovine
	CTCGCAGCAAAGCAAGATGTGTCTCAGAAGCTAACC		Murine
	978	1014	
Primer 2	GCCCTGCAGTTAGGTTCTGATCCAGGATGAAAATTTG		Human
	GTCCTGCAGTTAGGTTCTAACCTCAGTATGAAACCTC		Bovine
	TCCCTGCAGGGGTCCGATCCTAGGATGCAACGTTGGA		Murine

p35

	-20	16	
Primer 1	CCCTGCAGTGCCGGCTCAGCATGTGTCCAGCGCGCA		Human
	ATGTGCCCCGCTTCGCA		Bovine
	CCTCTCAGTGCCGGTCCAGCATGTGTCAATCACGCT		Murine
	650	686	
Primer 2	GAGTGACGAGCTATCTGAATGCTTCCTAAAAAGC.GA		Human
	GAATGATGAGCTATCTGAGTTCTTCCTAGAATGCTGA		Bovine
	GGGTGATGGGCTATCTGAGCTCCGCCTGAAAGCTCAA		Murine
	282	319	
Primer 3	TTGGAATTAACCAAGAATGAGAGTTGCCTAAATTCCAG		Human
	CTGGAATTAGCCACGAATGAGAGTTGCCTGGCTTCCAG		Bovine
	CTGGAACTACACAAGAACGAGAGTTGCCTGGCTACTAG		Murine
	544	581	
Primer 4	CAAAAATCCTCCCTTGAAGAACCGGATTTTTTATAAAAC		Human
	CAGAAACCCTCCCTGAAAGAACTGGATTTTTTACAAGAC		Bovine
	CAGAAACCTCCTGTGGGAGAAGCAGACCCTTACAGAGT		Murine

IL-18

	160	191	
Primer A	TTTGTCGCAGGAATAAAGATGGCTGCTGAACC		Human
	CCTCTTGGCCCAGGAACAATGGCTGCCATGTC		Murine
	750	784	
primer B	CGAAGACTAGCTATTAAATTTTCATGCCGGGCGCA		Human
	CTTACATCAAAGTTAGGTGGGGAGGGTTTGTGTTC		Murine
	229	260	
Primer C	TTTATTGACAATACGCTTTACTTTATAGCTGA		Human
	TTTATTGACAACACGCTTTACTTTATACCTGA		Murine
	725	259	
primer D	GATCTATAATGTTCACTGTTCAAAACGAAGACTAG		Human
	AATCTGTAATGTTCACTCTCACTAACTTACATCAA		Murine

Figure 4.6 Derivation of primers from published IL-12 and IL-18 sequences. In the case of p40 and p35 the numbers relate to nucleotide positions in the bovine sequence and for IL-18 in the human sequence.

For amplification of equine p35 all the primer sequences were derived from a consensus of the human and bovine equivalents. In the case of p40, primers 1, 3 and 4 are derived from human sequence whereas primer 2 was derived from the bovine sequence. Primers 3 and 4 were a gift from Dr. Mary B. Tompkins (details in Rottman *et al*, 1995). Table 4.2 shows the sequences of primers designed and figure 4.7 their relative position in the respective genes.

Table 4.2 Primers for PCR amplification of equine IL-12 and IL-18.

Target gene	Name	Orientation	Sequence (5'-3')	Tm
IL-12 p40	p40.1	sense	CAGAGCAAGATGTGTCACCAG	64°C
	p40.2	antisense	CATACTGAGGTTAGAACC	52°C
	p40.3	sense	AGATGCTGGCCAGTACACCT	62°C
	p40.4	antisense	GGGAGAAGTAGGAATGTGGAG	64°C
IL-12 p35	p35.1	sense	CAGTGCCGGCTCAGCATGTG	66°C
	p35.2	antisense	GCTTTT TAGGAAGCATT CAGATAGC	70°C
	p35.3	sense	CACGAATGAGAGTTGCCT	54°C
	p35.4	antisense	GAAGTATGCAGAGCTTGA	52°C
IL-18	A	sense	GCAGGAATAAAGATGGCTGC	60°C
	B	antisense	GGCATGAAATTTTAATAGCTA	56°C
	C	sense	GACAATACGCTTTACTTTAT	52°C
	D	antisense	GCGTTTTGAACAGTGAACAT	54°C

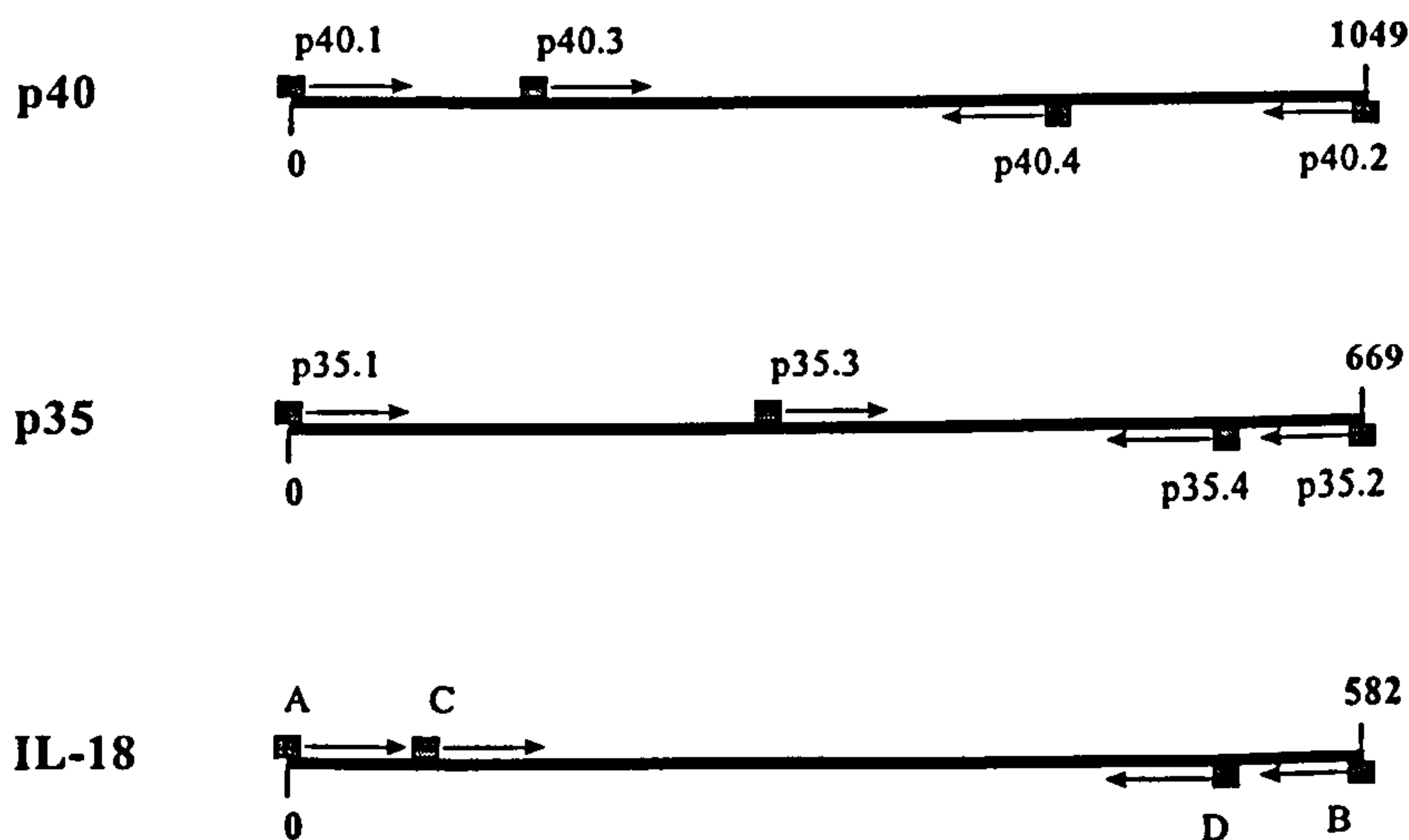


Figure 4.7 Relative positions of PCR primers in respective target sequences.

The primers were ordered for synthesis at MWG-biotech (Germany) and the corresponding melting temperatures (T_m) were calculated using the formula $T_m = 4x(G+C) + 2x(A+T)$.

Cycling conditions

For PCR amplification of eqIL-12 and eqIL-18 Perkin Elmer PCR core reagents were used. All primers were used at a final concentration of 1 μ M (2.5 μ l per reaction of each primer at 20 pmoles/ μ l) in a 50 μ l reaction volume with 2.5 units (0.5 μ l) of Taq polymerase, 5 μ l of 10X PCR buffer, 200 μ M dNTP mix (5 μ l of a solution containing 1.25 mM each dNTP) and 24.5 μ l of dH₂O. The template consisting of 10 μ l of cDNA per reaction was added just prior to PCR cycling. In the case of IL-18 secondary PCR reactions were also performed, using as template 1 μ l of the primary PCR reaction product. PCR reactions were performed in a Perkin Elmer 9600 thermal cycler with the following conditions. For both p35 and p40, 94°C for 5 min., followed by 30 cycles of 94°C for 1 min., 55°C for 1.5 min. and 72°C for 2 min., terminating with a single cycle of 72°C for 7 min. For IL-18 primary PCRs conditions were 94°C for 5 min with 30 cycles of 94°C for 45sec, 45°C 1 min and 72°C for 1.5 min terminating with 72°C for 7 min and for secondary PCRs 30 cycles of 94°C for 45sec, 45°C 1 min and 72°C for 2 min. In the case eqIL-12, two fragments of p40 were obtained by using primers p40.1 and p40.4 (5' fragment) and primers p40.3 and p40.2 (3' fragment) and p35 was also obtained in two fragments using p35.1 with p35.4 (5' fragment) and p35.3 with p35.2 (3' fragment). To obtain eqIL-18 in a single fragment an equine specific primer was design based on the PCR product sequence generated by primers A+D and C+B, this new primer, termed G (5'-GCTAGTTCTGGTTTTGAACA-3') successfully amplified the full length IL-18 cDNA in combination with primer A.

4.2.3 Cloning and sequencing of eqIL-12 and eqIL-18

PCR products were cloned into pCR-II TA cloning vector (Invitrogen) following the manufacturer instructions. Plasmid DNA was amplified by transformation and growth of the Inv α F' strain of *E. coli* (mat & meth). Recombinants were screened by

restriction enzyme digestion of minipreparations of plasmid DNA (mat & meth) using the enzymes *SpeI* and *NotI*. Double digestion using these enzymes, followed by electrophoresis in a 1.2% agarose gel stained with ethidium bromide (math & meth) generated the following band pattern: for p40 (5' fragment ~ 800 bp and 3' fragment ~750 bp); for p35 (5' fragment ~600bp and 3' fragment ~400bp) and for IL-18 a unique fragment of ~600 bp.

Fifteen p40 clones, 15 p35 clones and 10 IL-18 clones were subcultured by overnight growth in LB medium containing 100µg/ml of ampicillin at 37°C. Sequencing quality DNA was recovered using QIAprep Miniprep kit (Qiagen), observing the manufacturer instructions, analysed by ethidium bromide stained agarose electrophoresis and quantified by spectrophotometry. Sequencing reactions were prepared using the ThermoSequenase Cycle Sequencing Kit (Amersham) with vector derived M13 forward and reverse primers, modified by 5' labelling with the IRD-800 fluorochrome (MWG-Biotech). Sequencing reactions were run in a LICOR-400 automated sequencer (math & meth). Sequences generated for the clones across both strands were analysed by GCG software and consensus sequences for eqp40, eqp35 and eqIL-18 were generated.

4.2.4 Generation of full length eqIL-12 p40 and p35

Full length p35 and p40 cDNAs were obtained by cloning strategies that took advantage of unique restriction sites in the overlapping regions of both molecules, summarised in figures 4.8 and 4.9 for p40 and p35, respectively. The full length open reading frames obtained in this fashion were re-sequenced and found to be 100% conserved to the respective consensus sequences.

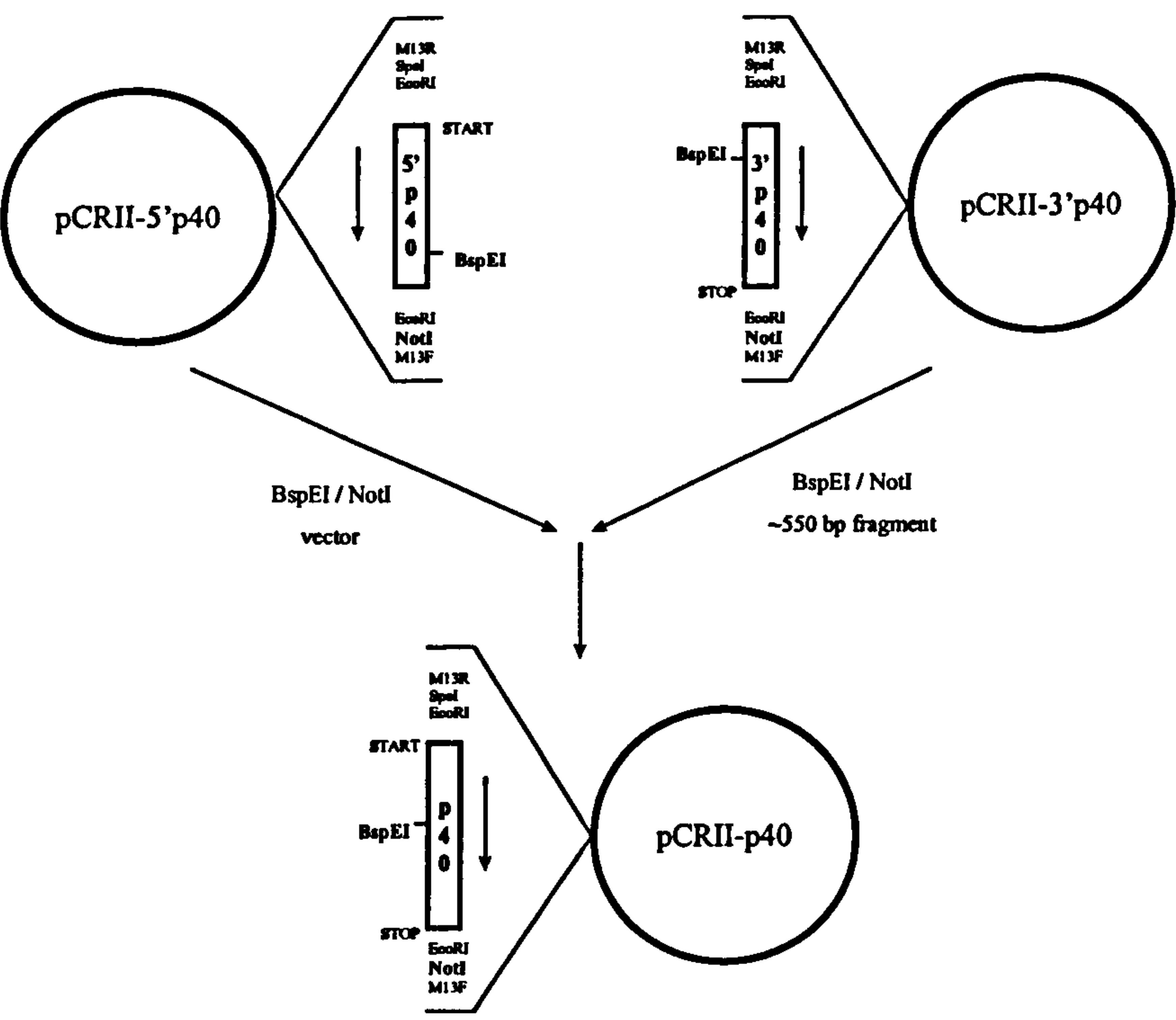


Figure 4.8 Generation of full length equine p40.

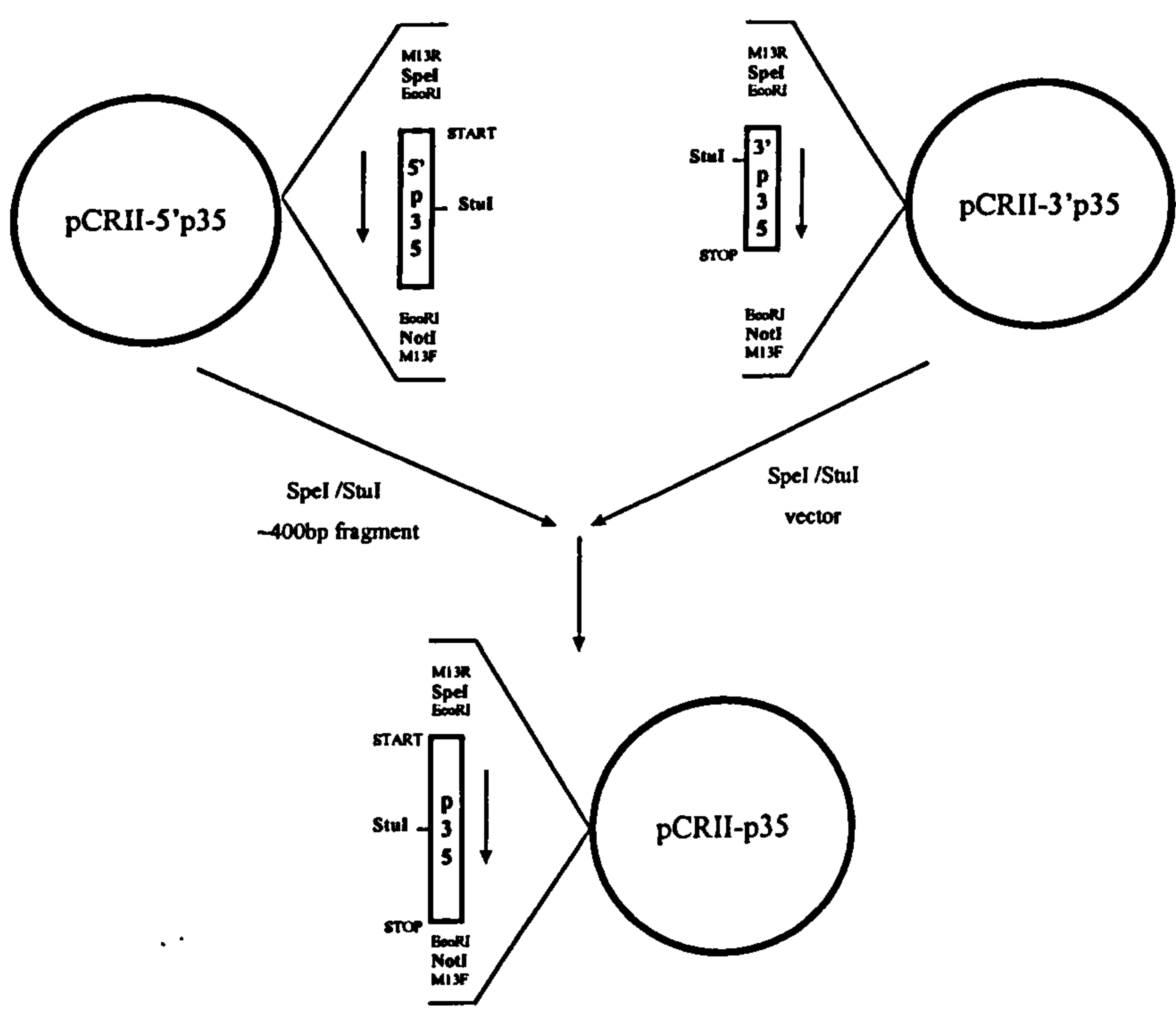


Figure 4.9 Generation of full length equine p35.

4.2.5 Cloning of eqIL-12 and eqIL-18 in mammalian expression vectors

The generation of constructs containing eqIL-12 and IL-18 for DNA vaccination utilised a plasmid suitable for driving the expression of these cytokines in mammalian cell systems. The plasmid chosen was the pCI-Neo (Promega) and the reasons for its selection are reviewed in chapter 6.

The secretion of bioactive recombinant IL-12 is only achieved by simultaneous expression of subunits p40 and p35 in the same cell. To ensure that both subunits would be delivered (by transfection) to individual cells, equine p40 and p35 were cloned into a single vector with dedicated gene promoters and polyadenylation signals driving expression of both mRNA species (figure 4.10). Additionally, in this system p40 expression is driven by a weaker promoter (lacking intronic sequence) than that regulating p35 expression, favouring secretion of heterodimeric IL-12 rather than monomeric p40, known to have receptor binding capability and therefore a competitive inhibitory effect over IL-12 activity.

Table 4.3 Nucleotide sequences used in the construction of eqIL-12 and eqIL-18 expression plasmids.

		Tm
IL12p40	MluI 5'primer 5'- GCACGCGTTCAGAGCAAGATGTG-3'	72°C
	3'primer 5'- GCTCTAGAGCATCCCTAACTGC-3'	68°C
	XbaI	
IL12p35	MluI 5'primer 5'- GCACGCGTTCGGCTTGGATATG -3'	70°C
	3'primer 5'- GCTCTAGACGGCTTGCTTTTtagg-3'	72°C
	XbaI	
IL-18	Bst1107I 5'primer 5'- GCACCATGGTATACTTTGGCAG-3'	66°C
	3'primer 5'- GCTAGTTCTGGTTTGAACA-3'	56°C
Linker* 5'-CTAGCATTTAAATATG—Ig leader sequence—GTTCCACTGGTGATATCATTTAAATG-3'		
NheI		EcoRV Sall

* Sequence derived from the plasmid pSecTag2 (Invitrogen).

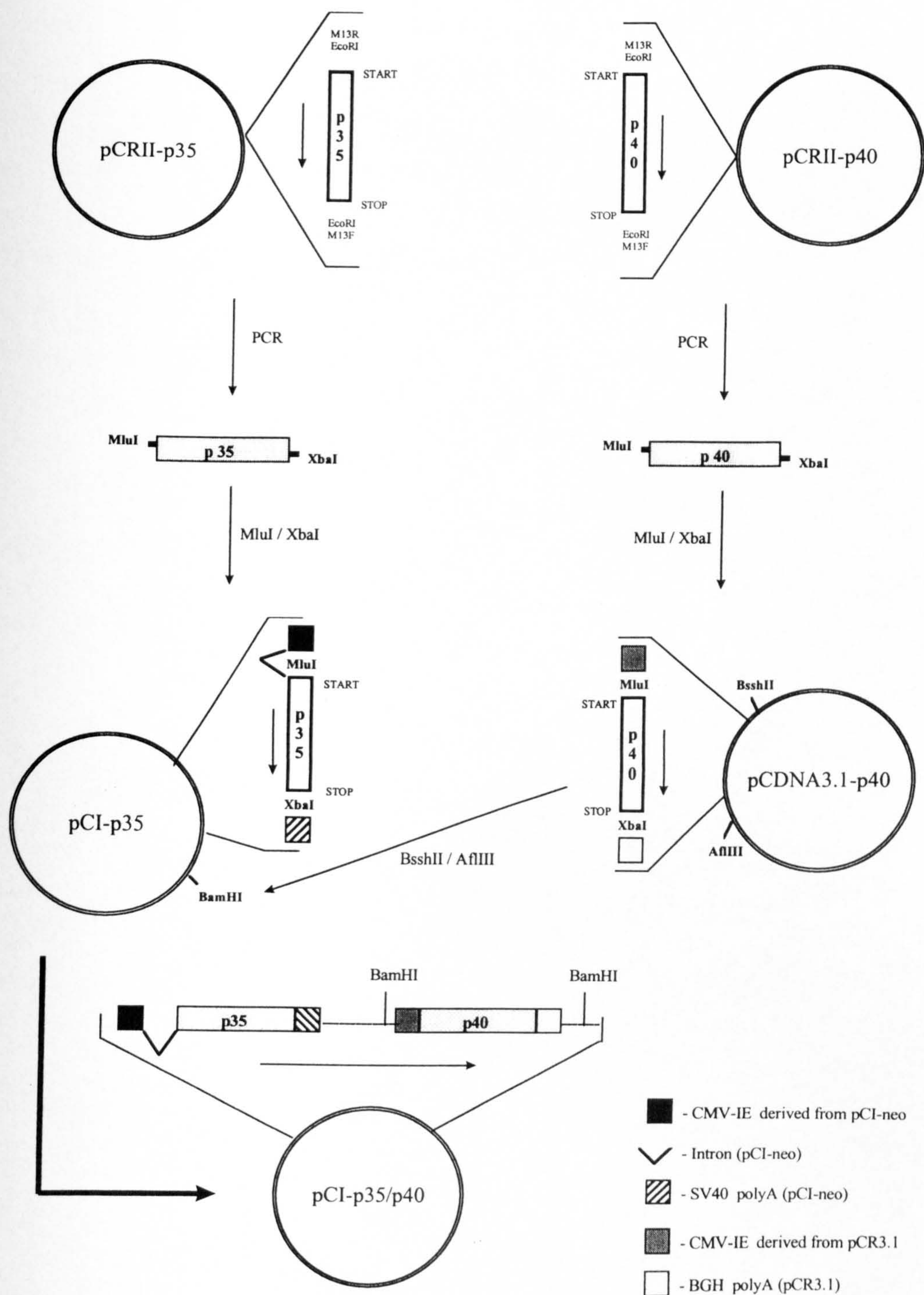


Figure 4.10 Strategy for construction of plasmid driving the expression of both p35 and p40 subunits of eqIL-12.

In summary equine p40 and p35 specific primers, tagged with restriction enzyme recognition sites (table 4.3) were used to amplify full length p40 and p35 from

plasmid template. Using the engineered enzyme sites (MluI and XbaI) p40 and p35 were cloned into the multiple cloning site of pCDNA3.1 (Invitrogen) and pCI-Neo, respectively. The p40 cDNA together with the pCDNA3.1 CMV promoter and polyadenylation signal was then removed from pCDNA3.1 using the enzymes BssHII and AflIII and slotted into the pCI-Neo vector using a BamHI site just downstream of the expression cassette CMV promoter-intron-eqp35-polyadenylation sequence.

In the case of eqIL-18, an expression plasmid was constructed by cloning equine mature IL-18 (mIL18) downstream of an artificial signal peptide sequence. Because this signal peptide is efficiently processed in transfected cells, it eliminates the need for the presence of ICE in the cells transfected by *in vivo* administration of DNA, that would otherwise be required for processing IL-18 precursor (proIL-18) with secretion of biologically active equine mIL-18.

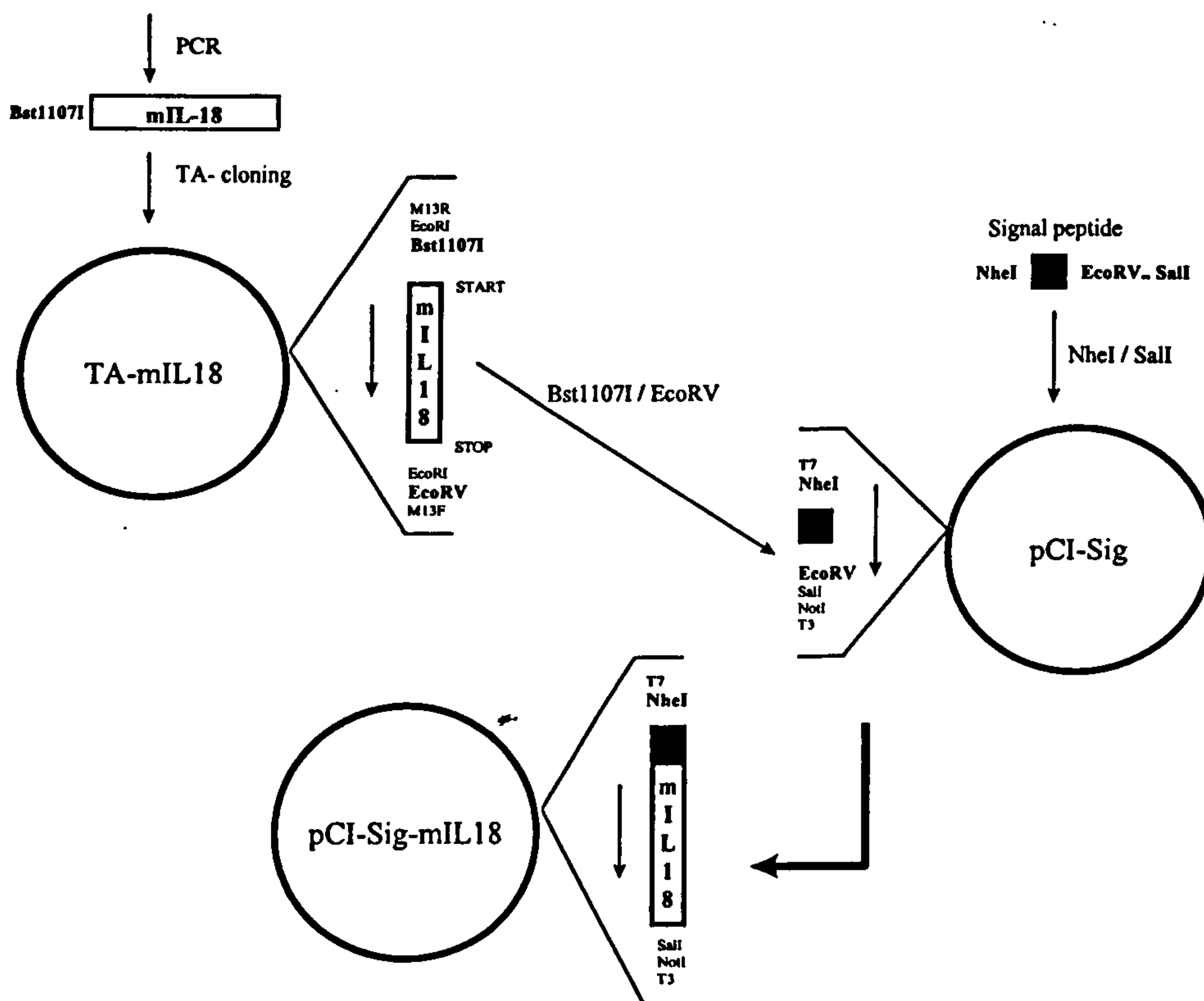


Figure 4.11 Strategy for construction of plasmid driving eqIL-18 expression linked to an artificial leader sequence.

Briefly, a linker sequence (table 4.3) containing an artificial signal peptide derived from the V-J2-C region of the mouse Ig kappa-chain, was synthesised and cloned into the *NheI* and *Sall* enzyme sites of pCI-Neo (pCI-Sig). Also, eqIL-18 specific primers (table 4.3) were used to amplify the mature portion of the molecule from plasmid DNA template. The 5' primer contained a *Bst1107I* enzyme site. The PCR product was cloned into a PCR-II TA cloning vector and excised from this plasmid using the *Bst1107I* site and an *EcoRV* site provided by the vector. The fragment generated was cloned into the *EcoRV* site of the artificial signal peptide generating the chimera pCI-sig-mIL18.

4.3 Results

Equine IL-12

As mentioned in the description of methods, both p35 and p40 were obtained in two overlapping fragments each (results not shown). To obtain the sequence data, 15 clones of p35 and 15 clones of p40 were sequenced across both strands and the resulting data was analysed to derive consensus sequences for the respective open reading frames.

Regarding the p40 subunit of equine IL-12, analysis of the sequence data revealed an open reading of 990 bp encoding a precursor polypeptide of 329 aminoacids (figure 4.12). The predicted peptide sequence has an identity of 86% towards human and bovine and 66% towards the corresponding murine sequence. Comparison of the equine p40 predicted protein with the human sequence revealed conservation of features such as 3 of the 4 consensus N-linked glycosylation sites, 9 of the 10 cysteine residues and the sequence $_{320}\text{WSEWAS}_{325}$ similar to the consensus WSXWS typical of the hemopoietin receptor family (figure 4.13). Further analysis of the antigenic structure of equine p40 (figure 4.18-A) disclosed the hydrophobic nature of the N-terminal region of the protein, typical of many cytokines, suggesting it has the same post-translational role in the efficient cellular trafficking and secretion of the molecule.

The equine p35 open reading frame is 669 bp in length and codes for a polypeptide of 222 aminoacids (figure 4.14). Equine p35 peptide shows a reasonable degree of homology to correspondent mammalian proteins with identities of 82, 80 and 59% to human, bovine and murine peptides, respectively. When compared to the human molecule shows a notable conservation of features, including all 3 predicted N-linked glycosylation sites and all 7 cysteine residues and three leucine zipper motifs thought to be involved in protein-protein interactions (figure 4.15). Again, antigenic analysis of the molecule, revealed a typical hydrophobic leader sequence (figure 4.18-B).

Equine IL-18

In relation to equine IL-18, the cloned gene fragment contains a full size open reading frame of 582 bp that codes for a 193 residue equine proIL-18 (figure 4.16). Sequence homology studies (figure 4.17) revealed that features in common with human IL-18 include the ICE processing site (Asp₃₆-Tyr₃₇) and those of caspase-3 (Asp₇₁-Ser₇₂ and Asp₇₆-Asn₇₇) and the IL-1-like signature sequence (F-X₁₂-F-X-S-X₆-F-L-) in residues 138 to 161. As in the case of mouse and human IL-18, the equine peptide is deprived of N-linked glycosylation sites. The overall identity of equine IL-18 peptide to the mouse and human representatives is of 67 and 79%, respectively. Analysis of the sequence antigenic structure (figure 4.18-C) revealed a non-hydrophobic and therefore unusual leader sequence typical of IL-18 and of the IL-1 family of proteins.

1 ATGTGTCACCAGTGGTTGGTCCTCTCCTGGTTTTCCCTGGTTTTGCTGGCGTCTCCCCTC **60**
M C H Q W L V L S W F S L V L L A S P L (20)

ATGGCCATATGGGAACTGGAGAAAGATGTGTATGTTGTAGAATTGGATTGGTACCCTGAT **120**
M A I W E L E K D V Y V V E L D W Y P D (40)

GCCCCTGGAGAAATGGTGGTCCTCACCTGCAATACCCCTGAAGAAGAAGGCATCACCTGG **180**
A P G E M V V L T C N T P E E E G I T W (60)

ACCTCGGCCCAGAGCAATGAGGTCTTAGGCTCTGGCAAAACCTTGACCATCCAAGTCAAA **240**
T S A Q S N E V L G S G K T L T I Q V K (80)

GAGTTTGGAGATGCTGGCTGGTACACCTGTCACAAAGGAGGCGAGGTTCTGAGCCATTCT **300**
E F G D A G W Y T C H K G G E V L S H S (100)

CACCTGCTGCTTCACAAGAAGGAAGATGGAATTTGGTCCACTGACATTTTAAAAGACCAG **360**
H L L L H K K E D G I W S T D I L K D Q (120)

AAAGAATCCAAAATAAGACCTTTCTAAAATGTGAGGCAAAGAATTAT**TCCGGAC**GTTTC **420**
K E S K N K T F L K C E A K N Y S G R F (140)

ACATGCTGGTGGCTGACAGCAATCAGTACTGATTTGAAATTCAGTGTCAAAGCAGCAGA **480**
T C W W L T A I S T D L K F S V K S S R (160)

GGTTCCTCTGACCCCCGAGGGGTGACGTGTGGAGCAGCGACACTCTCCGCAGAGAGGGTC **540**
G S S D P R G V T C G A A T L S A E R V (180)

AGCGTGGACGACAGGGAGTATAAGAAGTACACGGTGGAGTGTGAGGAGGGCAGTGCCTGC **600**
S V D D R E Y K K Y T V E C Q E G S A C (200)

CCGGCCGCCGAGGAGAGCCTGCCATTGAGATCGTGGTGGATGCTGTTCAAGCTCAAG **660**
P A A E E S L P I E I V V D A V H K L K (220)

TATGAAACTACACCAGCGGCTTCTTCATCAGGGACATCATCAAACCAG**G**ACCCACCCAAG **720**
Y E N Y T S G F F I R D I I K P D P P K (240)

AACCTGCAGCTGAAGCCATTAAAGAATTCTCGGCAGGTGGAGGTCAGCTGGGAGTACCCC **780**
N L Q L K P L K N S R Q V E V S W E Y P (260)

GAGACCTGGAGCACCCACATTCCTACTTCTCCCTGACATTCTCTATTGAGGTCCAGGGC **840**
E T W S T P H S Y F S L T F S I Q V Q G (280)

AAGAACAAGAAGGAAAGGAAAGACAGACTCTTCATGGATGAGACTTCAGCCACAGTCACA **900**
K N K K E R K D R L F M D E T S A T V T (300)

TGCCACAAGGATGGCCAGATCCGTGTCCAAGCCAGGGACCGCTACTACAGCTCATCCTGG **960**
C H K D G Q I R V Q A R D R Y Y S S S W (320)

AGCGAATGGGCATCCGTATCCTGCAGTTAGGGATGCAGACTCAGGCAGCCCAGGCCAGAC **1020**
S E W A S V S C S * (329)

CTGAACACTCAGTGTACCCAGGTTCTAACCTCAGTATG **1058**

Figure 4.12 Equine IL-12 p40 subunit nucleotide sequence and predicted a.a. sequence. Nucleotides are numbered in bold type and a.a. in brackets. Primer determined sequence is underlined. The restriction site for the enzyme BspEI, used for recovery of full length p40, is shadowed. An allelic difference (A or G) observed at base 714, that does not incur in substitution of the coded amino acid (D-aspartic acid) is also shadowed.

	1				50
Equine p40	MCHQWLVL	SW FSLVLLASPL	MAIWELEKDV	YVVELDWYPD	APGEMVVLTC
Bovine p40	-HP-Q--V--	-----I	V-M-----N-	-----	----T-----
Human p40	----Q--I--	----F-----	V-----K----	-----	-----
Murine p40	--P-K-TI--	-AI---V----	--M-----	----V--T--	----T-N----
	51				100
Equine p40	NTPEEEGITW	TSAQSNEVLG	SGKTLTIQVK	EFGDAGWYT	C HKGGEVLSHS
Bovine p40	D----D----	--D--S----	-----	-----Q----	-----A--R-
Human p40	D----D----	-LD--S----	-----	-----Q----	-----
Murine p40	D----DD----	--D-RHG----	-----T--	--L--Q----	-----T----
	101				150
Equine p40	HLLLHKKEDG	IWSTDILKDQ	KESKNKTFLK	CEAKNYSGRF	TCWWLTAIST
Bovine p40	L-----	-----	--P-A-S----	----D--H-	-----
Human p40	L-----	-----	--P-----R	-----	-----T----
Murine p40	-----N-	----E--NF	***-----	---P-----	--S--VQRNM
	151				200
Equine p40	DLKFSVKSSR	GSSDPRGVT	C GAATLSAERV	SVDDREYKKY	TVECQEGSAC
Bovine p40	-----	-----	---L----K-	-LEH---N--	-----
Human p40	--T-----	-----Q----	-----	RG-NK--*E-	S-----D----
Murine p40	----NI---S	S-P-S-A---	-M-S----K-	TL-Q-D-E--	S-S---DVT-
	201				250
Equine p40	PAAEESLP	IE IVVDAVHKLK	YENYTS	SGFFI RDI	IKPDPPK NLQLKPLKNS
Bovine p40	-----L--	V--E-----	-----S----	-----	----R-----
Human p40	-----	VM-----	-----S----	-----	-----
Murine p40	-T---T----	LAL	E-RQQN-	----STS----	-----M-----
	251				292
Equine p40	RQVEVSWEYP	ETWSTPHSYF	SLTFSIQVQG	KNKKER****	****KDRLFM
Bovine p40	-----	D-----	----CV----	---R-K----	-----**----
Human p40	-----	D-----	----CV----	-S-R-K----	-----V-T
Murine p40	*-----	DS-----	--KFFVRI-R	-KE-MKETEE	G
	293				329
Equine p40	DETSATVT	CH KDGQIRVQAR	DRYSSSWSE	WASVSCS	
Bovine p40	-QT--K----	--ANV-----	-----F----	-----	86%
Human p40	-K-----I-R	-NAS-S-R-Q	-----	----P--	86%
Murine p40	EK--TE-Q-*	-G-NVC---Q	----N--C-K	--C-P-RVRS	66.1%

Figure 4.13 Sequence comparison between equine p40 subunit of IL-12 and other published mammalian species, highlighting a.a substitutions. Homology percentages in relation to the equine sequence are shown. Numbers correspond to a.a. positions of equine sequence. The predicted signal peptide sequence is shaded blue. Conserved N-linked glycosylation sites are underlined and the haemopoietin receptor related sequence is in bold type. (*) represent absent residues.

1 ATGTGCCCCGCGCGCGGCCTCCTCCTTGTGGCCATCCTGGTCCTCCTAAACCACCTGGAC **60**
M C P P R G L L L V A I L V L L N H L D (20)

CACCTCAGTTTGGCCAGGAACCTCCCCACAGCCACACCAGGCCAGGAATGTTCCAGTGC **120**
H L S L A R N L P T A T P G P G M F Q C (40)

CTCAACCACTCCCAAAACCTGCTGAGGACCGTCAGCAACACGCTTCAGAAGGCCAGGCAA **180**
L N H S Q N L L R T V S N T L Q K A R Q (60)

ACCCTAGAATTCTACTCCTGCACTTCTGAAGAGATCGATCATGAGGATATCACAAAAGAC **240**
T L E F Y S C T S E E I D H E D I T K D (80)

AAGAGCAGCACCGTGGCGGCCTGCCTCCCCCTGGAACCTGCCCCGAACGAGAGTTGCCTG **300**
K S S T V A A C L P L E L A P N E S C L (100)

GCTTCCAGAGAGATCTCTTTCATAACTAATGGGAGTTGCCTGACCCCCGGAAGGCCTCT **360**
A S R E I S F I T N G S C L T P G K A S (120)

TCTATGATGACGCTGTGCCTTAGCAGCATCTATGAGGACTTGAAGATGTACCAGGTGGAG **420**
S M M T L C L S S I Y E D L K M Y Q V E (140)

TTCAAGGCCATGAATGCCAAGCTGTTGATAGATCCTCAGAGGCAGATCTTTCTGGATGAG **480**
F K A M N A K L L I D P Q R Q I F L D E (160)

AACATGCTGACAGCCATTGACAAGCTGATGCAGGCCCTGAACTTCAACAGTGAGACTGTG **540**
N M L T A I D K L M Q A L N F N S E T V (180)

CCACAAAAGCCCTCCCTTGAAGGACTGGATTTTTATAAACTAAAGTCAAGCTCTGCATC **600**
P Q K P S L E G L D F Y K T K V K L C I (200)

CTTCTTCATGCCTTCAGAATCCGCGCAGTGACCATCAACAGGATGATGGGCTATCTGAAT **660**
L L H A F R I R A V T I N R M M G Y L N (220)

GCTTCCTAA **669**
A S * (222)

Figure 4.14 Nucleotide and derived a.a. sequence for equine IL-12 p35 subunit. Numbers in bold type for nucleotide and in brackets for a.a. positions. Primer determined sequence is underlined. The recognition site for *StuI*, used to produced full length p35 is shadowed.

	-34						16
Equine p35						MCPPRG LLLVAILVLL	
Bovine p35						---L-S ---IST---	
Human p35	MWPPGSASQP	PPSPAAATGL	HPAARPVSLQ	CRLS---	A-S	-----TL--*	
Murine p35						--QS-Y --FLAT-A--	
	17						66
Equine p35	NHLDHL SLAR	NLPTATPGPG	MFQ CL NHSQN	LL RTVSNT LQ	KARQT L EFGYS		
Bovine p35	H--P-----G-	S---T-AS--	R*S--DY---	---A-----	-----		
Human p35	**-----	---V---D--	--P--H----	---A---M--	-----P		
Murine p35	-***-----	VI-VS****G	PARC-SQ-R-	--K-TDDMVK	T--EK-KH--		
	67						116
Equine p35	C TSEEIDHED	ITKDKSSTVA	A CLPLE L APN	E S C LASREIS	F ITNG S C LTP		
Bovine p35	-----	-----T---E	-----T-	-----T-	-----H--AS		
Human p35	-----	-----T---E	-----TK-	-----N-----	-----AS		
Murine p35	--A-D-----	--R-QT-T--	-----	-----T--T-	ST-R----P-		
	117						166
Equine p35	GKASSMMTL C	L SSIIYED L KM	YQVE F KAMNA	K L LIDPQRQI	FLDENMLTAI		
Bovine p35	--T-F-T---	-R-----	-H---Q----	---M--K---	---Q---A--		
Human p35	R-T-F--A--	-----	-----T---	---M--K---	---Q---AV-		
Murine p35	Q-T-L-----	-G-----	--T--Q-I--	A-QNHNHQ--	I--KG--V--		
	167						216
Equine p35	DKLMQALNFN	SETVPQKPSL	EGLDFYKTKV	K L C ILLHAFR	IRAVTINRMM		
Bovine p35	AE-----D	-----	KE-----	-----	-----D---		
Human p35	-E-----	-----S--	-EP-----I	-----	-----D-V-		
Murine p35	-E---S--H-	G--LR---PV	GEA-P-RV-M	-----S	T-V-----V-		
	222						
Equine p35	GYLNAS						
Bovine p35	S--SS-	80.2%					
Human p35	S-----	82.4%					
Murine p35	---SSA	58.6%					

Figure 4.15 Comparison of the predicted a.a. sequence of equine IL-12 p35 subunit with its counterpart of other animal species, showing the percentage of homology to the equine molecule. Numbers relate to a.a. positions in the equine sequence. Conserved cysteine residues (shadowed), N-linked glycosylation sites (underlined) and leucine zipper motifs (overlined) are shown. The typical hydrophobic leader sequence is shaded blue. Translation of the human molecule from the upstream initiation site results in the addition of N-terminal residues (positions -34 to 1)

1 ATGGCTGCTGGACCAGTAGAAGACAATTGCATTAGCTTGGTGGAAATGAAATTTATTGAC **60**
M A A G P V E D N C I S L V E M K F I D (20)
AACACACTTTACTTTGTAGCTGAAAACGATGAAAACCTGGAATCAGATTACTTTGGCAGG **120**
N T L Y F V A E N D E N L E S D Y F G R (40)
CTTGAACCTAAACTCTCAATCATACGAAATTTGAACGACCAAGTTCTCTTCATTAACCAG **180**
L E P K L S I I R N L N D Q V L F I N Q (60)
GGAAATCAACCTGTGTTTGAGGATATGCCTGATTCTGATTGTACAGACAACGCACCCCAG **240**
G N Q P V F E D M P D S D C T D N A P Q (80)
ACCGTATTTATCATATATATGTATAAAGATAGCCTCACTAGAGGTCTAGCGGTAACCATC **300**
T V F I I Y M Y K D S L T R G L A V T I (100)
TCTGTGAAGTGTGAGAAAACGTCTACTCTCTCCTGTAAGAACAAAATTATTTCTTTAAG **360**
S V K C E K T S T L S C K N K I I S F K (120)
GAAATGAGTCCTCCTGAGAATATCAATGATGAAGGAAATGACATCATATTCTTTCAGAGA **420**
E M S P P E N I N D E G N D I I F F Q R (140)
AGTGTTCAGGACATGATGATAAGATAACGTTTGAGTCTTCACTGTATAAAGGATACTTT **480**
S V P G H D D K I Q F E S S L Y K G Y F (160)
CTAGCTTGTGAAAAAGAGAATGATCTTTTCAAACCTCATTTTGAAAGAAAAGGATGAAAAT **540**
L A C E K E N D L F K L I L K E K D E N (180)
GGGGATAAATCTGTAATGTTCACTGTTCAAAACCAGAACTAG **582**
G D K S V M F T V Q N Q N * (193)

Figure 4.16 Equine IL-18 nucleotide sequence and predicted a. a. sequence. Numbers in bold correspond to nucleotide positions and a.a. are numbered in brackets. Primer determined sequence is underlined.

	1				↓		50
Equine IL18	MAAGPVEDNC	ISLVEMKFID	NTLYFVAEND	ENLESDYFGR	LEPKLSIIRN		
Human IL18	---E-----	-NF-A-----	-----I--D-	-----K	--S---V---		
Murine IL18	M--MS*EDS-	VNFK--M---	-----IP-EN	GD-----N---	-HCTTAV---		
	51		↓ ↓				100
Equine IL18	LNDQVLFINQ	GNQPVFEDMP	DSDCTDNAPQ	TVFIIYMYKD	SLTRGLAVTI		
Human IL18	-----D-	--RPL-----T	----R----R	-I---S-----	-QP--M----		
Murine IL18	I-----VDK	*R-----T	-I-QSASE--	-RL-----	-EV-----L		
	101						150
Equine IL18	SVKCEKTSTL	SCKNKIISFK	EMSPPENIND	EGNDIIFFQR	SVPGHDDKIQ		
Human IL18	-----I---	--E-----	--N--D--K-	TKS-----	-----N-M-		
Murine IL18	---DS-M---	-----E	--D-----D-	IQS-L----K	R----*N-ME		
	151						193
Equine IL18	FESSLYKGYF	LACEKENDLF	KLILKEKDEN	GDKSVMFTVQ	NQN		
Human IL18	----S-E---	-----R---	-----KE--L	--RSI-----	-ED	79%	
Murine IL18	-----E-H-	---Q--D-A-	-----K----	-----LT	-HQS	67%	

Figure 4.17 Comparison of the predicted amino acid sequence of eqIL-18 with other known IL-18 sequences, showing the percentage of homology to the equine protein and non-conserved residues. Proteolytic processing sites for ICE (↓) and caspase-3 (↓) are indicated and the IL-1-like signature sequence is shadowed. Numbers correspond to a.a. positions.

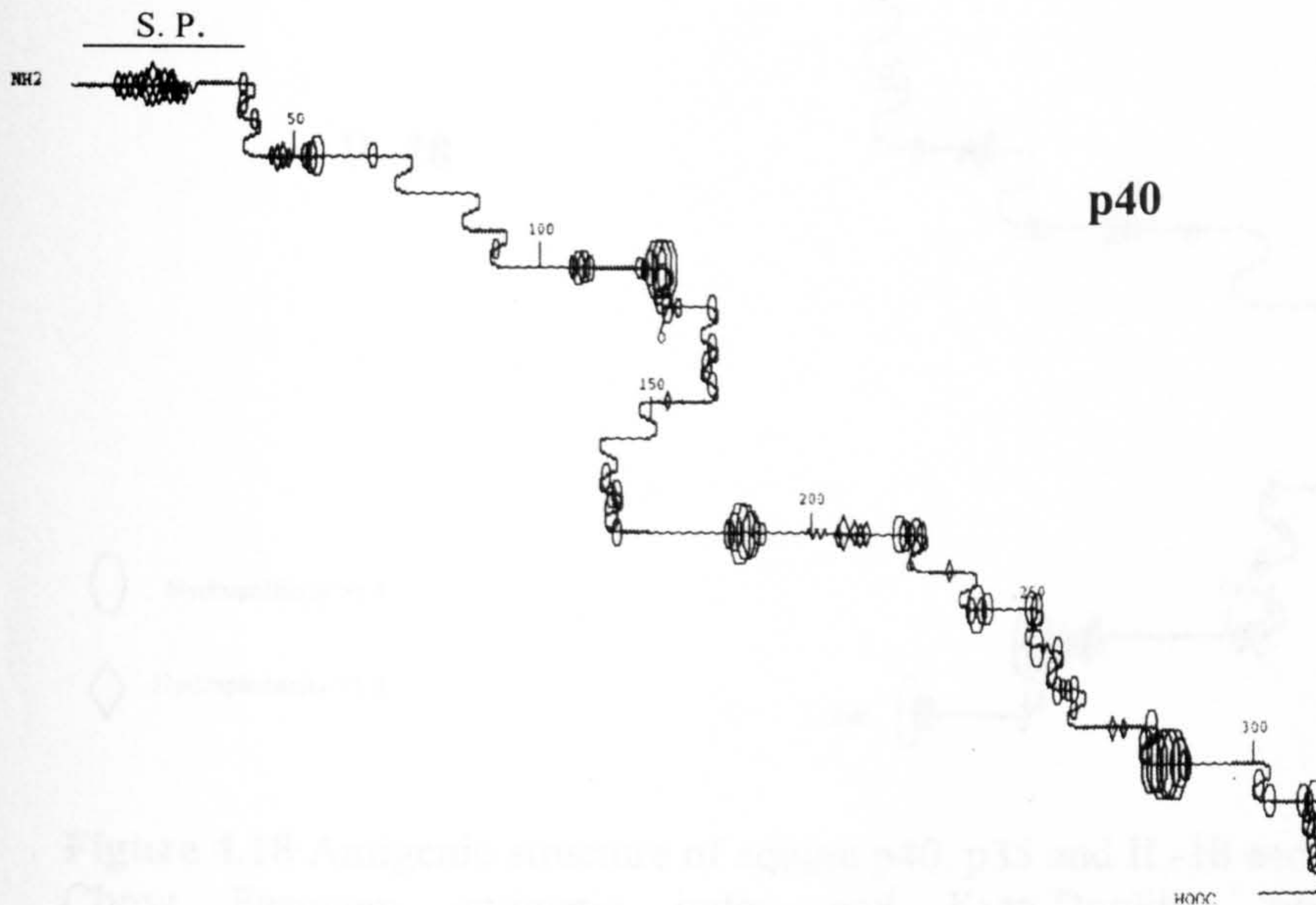
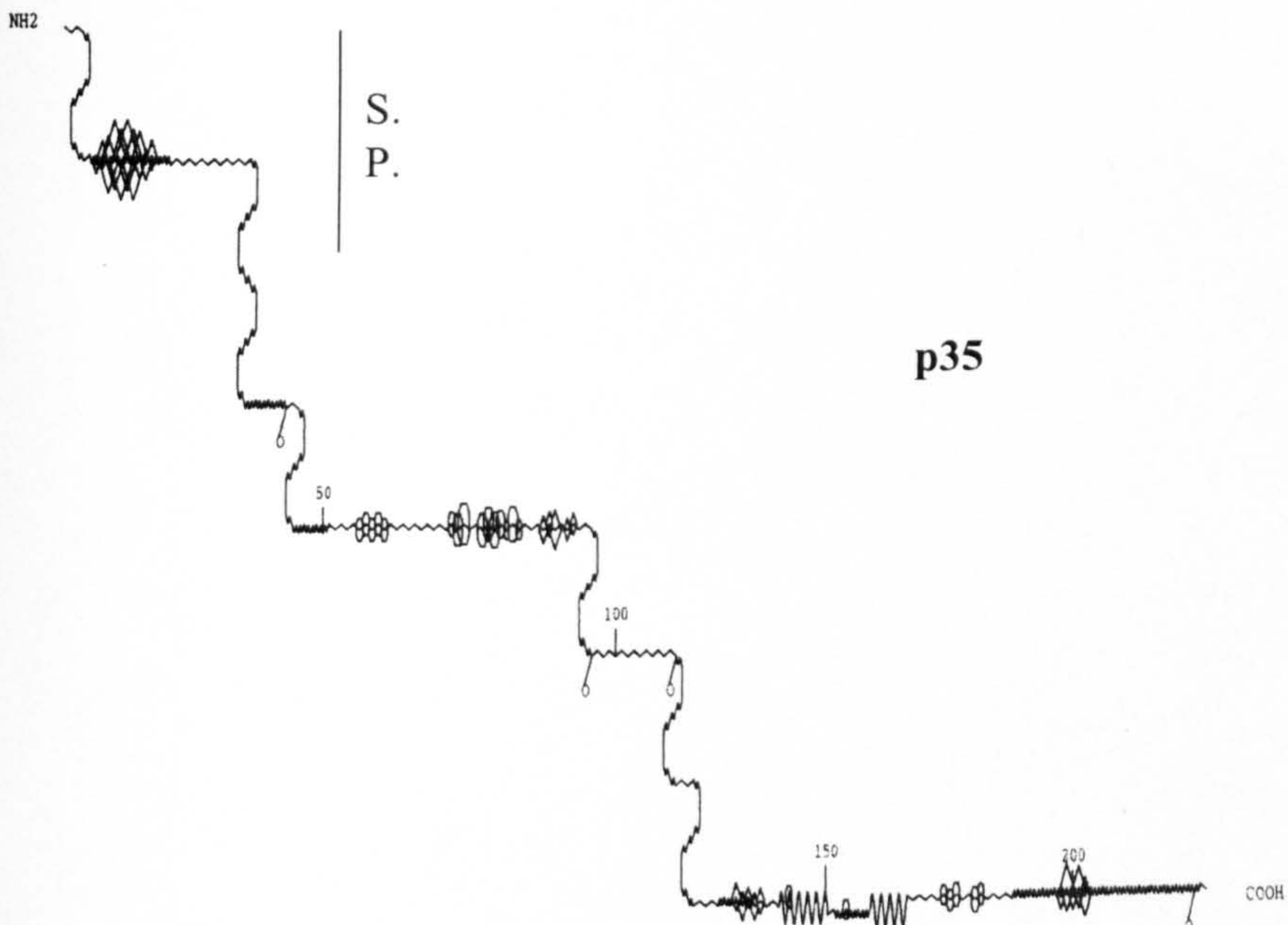


Figure 1.18 Secondary structure of p40, p35 and p18 as determined by the Chou-Fasman secondary structure index and Kyte-Doolittle prediction of hydrophobicity. Regions corresponding to the predicted signal peptides (S.P.) are identical.



4.4 Discussion

The aim of this part of the project was to clone the cDNAs of equine IL-12 and IL-18, to facilitate the investigation of their potential to drive antiviral responses *in vivo* through induction of IFN- γ production. The conservation of features observed in the nucleotide sequence and predicted amino acid sequence of both molecules, when compared to their counterparts of other mammalian species, allows for the conclusion that equine IL-12 and IL-18 were successively cloned.

The cloning of the cDNAs for equine IL-12 and IL-18 also facilitates their use in the production of the corresponding recombinant proteins. The expression of these proteins *in vitro* will permit the generation of bioactive molecules and antibodies against them, which are vital tools to attempt the dissection of the biology of these cytokines in the horse model, keeping in line with recent developments in basic immunology.

Preliminary work on expression of these cytokines has been carried out mainly as a means to verify the functionality of constructs to be used in the vaccine trial. So far transient expression of IL-12 (in single plasmid) has been accomplished and the expression efficiency assessed by northern blot. A mature (truncated) form of IL-18 has been expressed in *E. coli* and used as an immunogen for polyclonal antibody production. These antibodies were found to recognise eqIL-18 in cell lysates of transiently transfected mammalian cells, by western blot, and to cross-react with canine and feline IL-18.

Attempts are also being made to assess biological activity of these cytokines. Concerning IL-12 an assay to measure IL-12-induced proliferation of equine PBLs is presently in development, following success obtained with a similar assay for IFN- γ (chapter 3) and an encouraging report that eqIL-12 expressed in baculovirus was able to promote proliferation of phytohaemagglutinin (PHA) stimulated equine PBLs (Dr. Virgil E. Schijns, personal communication). In regard to IL-18, a first attempt was made to detect biological activity as measured by the upregulation of IFN- γ production. The assay, described by Konishi *et al* (1997), makes use of a human

myelomonocytic cell line that, in response to human IL-18, produces IFN- γ in a dose dependent manner. The authors also found the cell line to be responsive to murine IL-18, although acknowledging that the later was near 100-fold less potent. While we were able to repeat the author's results in regard to commercially available recombinant human IL-18 (R&D Systems) we were unable to detect specific activity of eqIL-18. We could also not detect eqIL-18 protein (by western blot) in the supernatants of transfected cells. Failure to detect eqIL-18 bioactivity could therefore be due to the presence of low amounts of bioactive IL-18 in the supernatants tested, low cross-reactivity of equine IL-18 in the human cell system and/or deficient processing and secretion of active mature IL-18 mediated by the artificial signal peptide. The latest is reinforced by reports that IL-18 is rendered active only after processing by ICE (Okamura *et al*, 1998). Taking this into consideration we have cloned the equine ICE cDNA (Wardlow *et al*, 1999 - Appendix I) and its role in processing the eqIL-18 precursor (equine proIL-18) is currently being assessed.

CHAPTER 5

CLONING AND EXPRESSION OF EQUINE INFLUENZA VIRUS GENES

5.1 Introduction

Influenza viruses cause the contagious and acute respiratory illness known as influenza. Epidemic and pandemic outbreaks in humans are documented as far back as 412 B.C. (Hippocrates) and have occurred at irregular intervals varying in severity. Major human pandemics include the 1889 Asiatic influenza, with a further outbreak in 1900 and the 1918 Spanish influenza that resulted in over 20 million deaths worldwide. The isolation of the first influenza virus from pigs in 1931, followed by human influenza virus in 1933 allowed the close monitoring of outbreaks and seroepidemiological studies that followed suggest that epidemics are caused by two main mechanisms: the introduction of new viral variants into human populations, usually from reservoir species (e.g. ducks) as happened in the epidemic of 1968 and the recirculation of viral strains thought to have been cleared from human populations as in 1957 and 1977 (Webster *et al*, 1983; Assad *et al*, 1983).

Influenza viruses belong to the family *Orthomyxoviridae* which is divided into three main genus (or types) on the basis of antigenic differences between major internal proteins, the nucleocapsid and matrix proteins. These viral types are organised into: influenza A, B and C , (Russell and Edington, 1985). Type A viruses are widely distributed, naturally infecting humans and other mammals including swine, horses and seals and a great variety of domestic and wild birds. Type B and C viruses infect predominantly humans although type C has been isolated from pigs in China (Guo *et al*, 1983). A further type, D, of tickborne viruses was described as being genetically and structurally similar to the other types (reviewed in Lamb and Krug, 1996). Within a viral type, strains are grouped according to the antigenic nature of the surface glycoproteins, haemagglutinin and neuraminidase.

A precise nomenclature system, last revised in 1980 (Hinshaw *et al*, 1983), has been devised by the World Health Organisation to facilitate close monitoring of outbreaks. Strains are defined by type (A to C), host, geographical origin, strain or laboratory reference number and year of isolation. The subtyping based on the nature of

haemagglutinin and neuraminidase is given in brackets [e.g., A/Swine/Wisconsin/5/76 (H1N1)].

Influenza viruses are subjected to a constant selective pressure to overcome rising immunocompetence in human and animal host populations (Killbourne, 1983). It is therefore hardly surprising that a wealth of subtypes of virus has been isolated from different host species, since the immunological pressure, in the shape of neutralizing antibodies, is mainly directed to the surface haemagglutinin and neuraminidase proteins, (Smith and Palese, 1989)

5.1.1 The virus

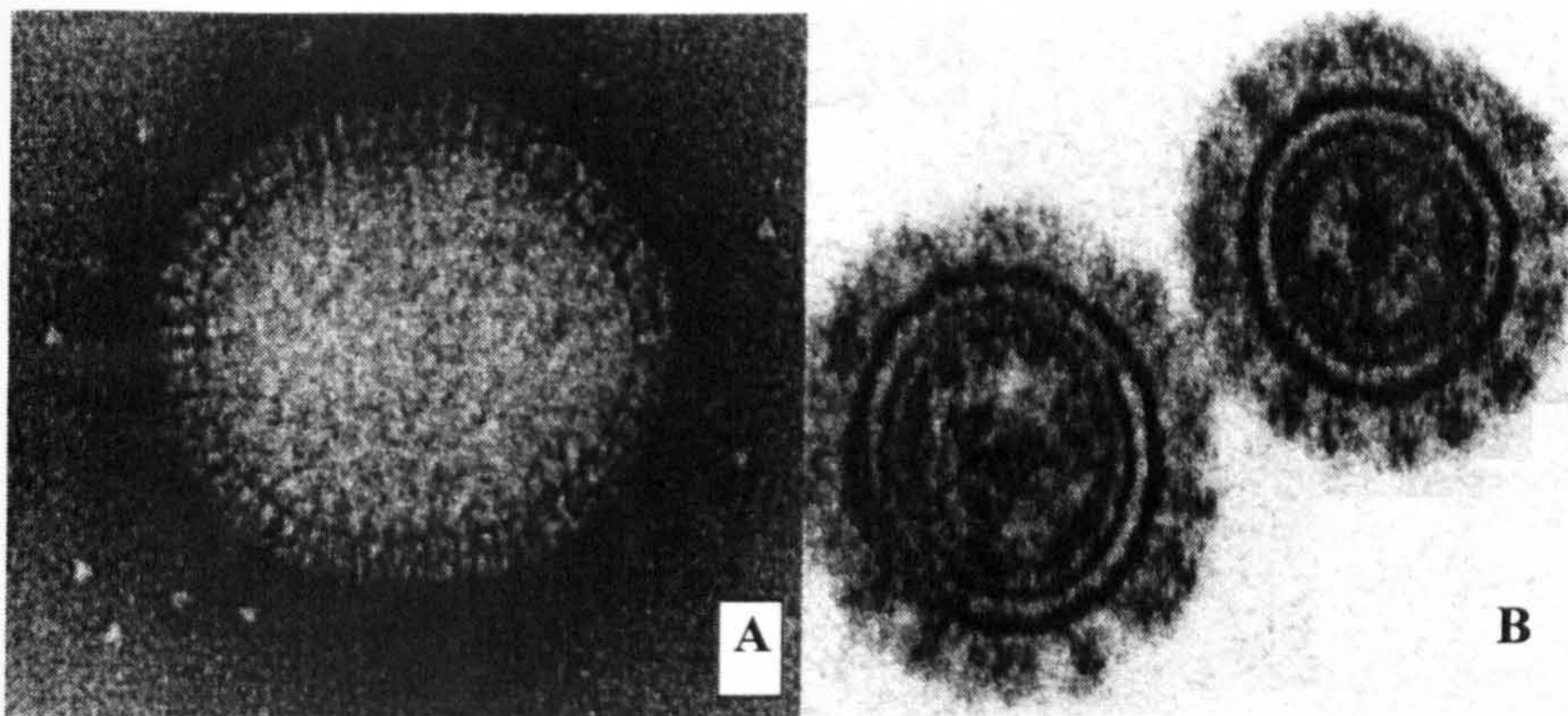


Figure 5.1 Influenza A virus. (A) negative staining electron microscopy (X27 000) and (B) detail (X300 000) showing virion components: spikes, viral membrane and internal RNA-protein elements (Oxford and Hockley, 1987).

Virion structure

The virion of influenza A has a regular spherical appearance (figure 5.1) with 80 to 120 nm in diameter, comprising the following structures (figure 5.2). A lipid envelope (derived from the host cell plasma membrane) from which radiate surface projections (spikes) formed by trimers of haemagglutinin (rod-shaped) and tetramers of neuraminidase (mushroom-shaped). A third transmembrane protein (M2) forms a functional pore. Underlining the lipid bilayer is the viral matrix formed by M₁ and NS₂ proteins. Inside

the virus are the RNP structures containing eight segments of RNA of different sizes (table 5.1). These structures consist of RNA and protein complexes where nucleocapsid protein is predominant. Also associated with RNP are the three polymerase proteins (PB₁, PB₂ and PA).

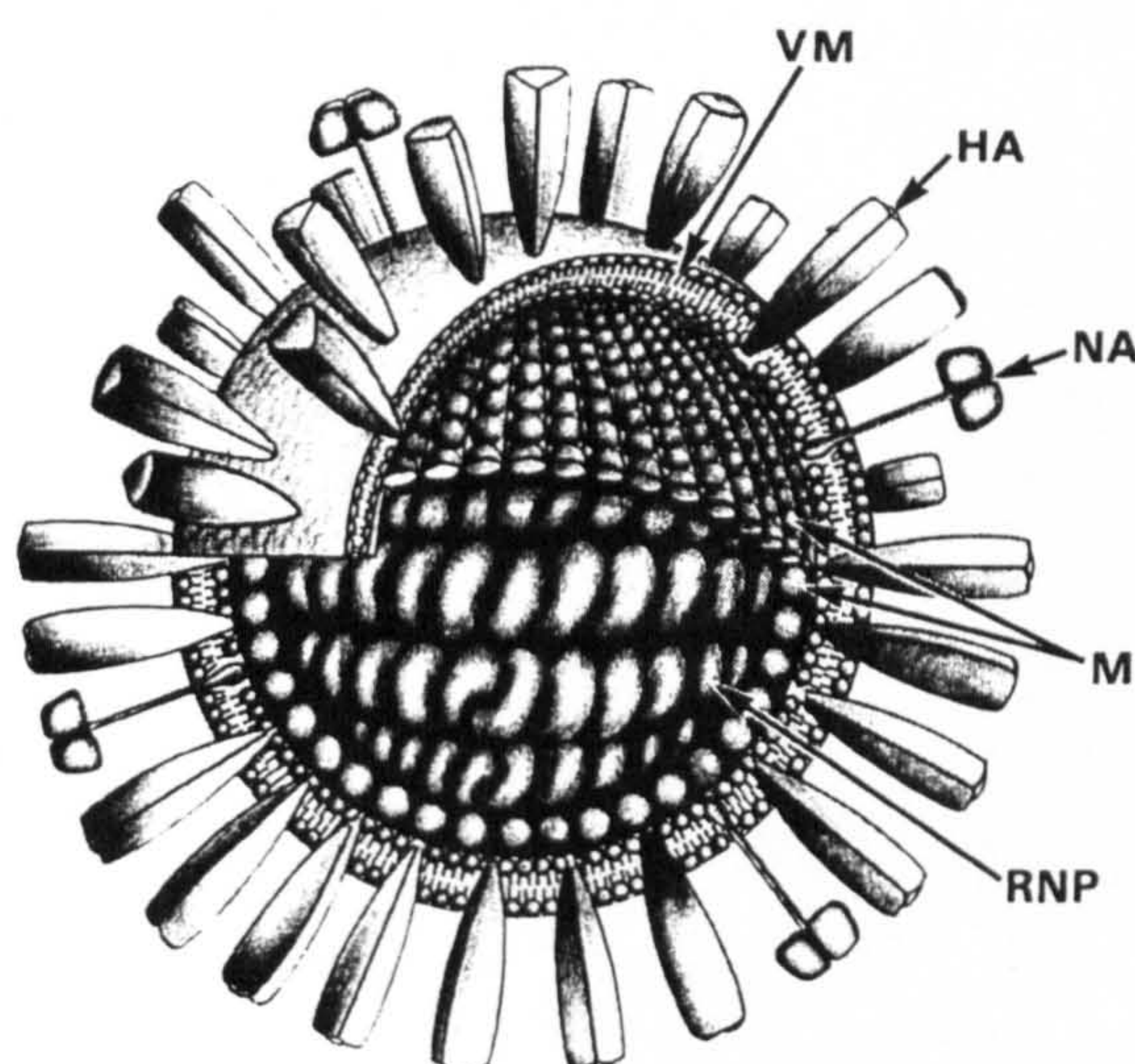


Figure 5.2 Diagram of influenza virus showing tridimensional relationships between the viral membrane and the spikes (HA and NA) forming the viral envelope, while M protein and RNP associate to form the viral core (Oxford and Hockley, 1987).

Genome

The Influenza A virus genome is composed of eight segments (table 5.1) of single negative stranded RNA [RNA(-)]. Transcription of the RNA(-) gives rise to mRNAs (by definition mRNAs are positive stranded) required for the synthesis of viral proteins. In addition, synthesis of antigenome, RNA(+), is a prerequisite for the replication of the virus as the RNA(+) will serve as template for further copies of genomic RNA(-).

Transcription and replication are performed by RNA-dependent RNA polymerases encoded and packaged by the virus, and take place in the nucleus of infected cells. Mechanisms such as overlapping reading frames, bicistronic mRNAs and coupled

translation of tandem cistrons are used as a means of maximising the coding capacity of the compact genome (reviewed in Lamb and Krug, 1996).

Table 5.1 Influenza A genome and encoded proteins.

RNA segment	Gene length (in nucleotides)	Encoded protein	Protein length (in a.a.)
1	2341	PB2	759
2	2341	PB1	757
3	2233	PA	716
4	1778	HA	566
5	1565	NP	498
6	1413	NA	454
7	1027	M1/M2	252/97
8	890	NS1/NS2	230/121

Note: a.a. - amino acids

Viral proteins

a) RNA segments 1, 2 and 3 are the largest in the influenza genomes and encode the three polymerases: PB1, PB2 and PA so called by their migration pattern in isoelectric focusing gels (PB1 and PB2 are basic and PA is acidic).

The P proteins are synthesised in the cytoplasm of the infected cell and then translocated to the nucleus possibly as a complex. Once in the nucleus the P complex is involved in transcription of viral mRNAs and in replication of RNA(-). In short, the PB₂ portion of the complex binds to capped RNA primers (derived from the host cell) containing the sequence m⁷GpppNm. Binding of this complex to the 3' end of RNA(-) leads to endonuclease activity rendering a mature transcription complex which is the substrate for initiation/elongation of transcripts by PB₁. It is not known which protein(s) in the complex are responsible for cleavage of mature complexes and for polyadenylation of mRNAs (Lamb and Krug, 1996).

b) RNA segment 4 encodes the haemagglutinin (HA) glycoprotein. HA is synthesised as a precursor polypeptide (562 to 566 a.a.), containing a N-terminal hydrophobic signal peptide (16 a.a.) which is cleaved from HA following insertion in the rough endoplasmic reticulum. This cleavage gives rise to a mature protein consisting of two portions HA₁ (319 to 326 a.a.) and HA₂ (221 to 222 a.a.) separated by a proteolytic cleavage site. Mature HA monomers associate non covalently to form functional HA trimers. The trimer spike is composed of a long fibrous stem (HA₂) extending from the lipid bilayer connected by a disulfide bond to the HA₁ globular domain. The N-terminal portion of HA₂ contains a hydrophobic motif termed fusion peptide. Six N-linked carbohydrate chains are attached to HA₁ and one chain to HA₂. These are likely to be needed for correct folding of the HA molecule in its native form (Skehel *et al*, 1994).

HA has three major functions in the replicative cycle of influenza virus (Ada and Jones, 1986; Lamb, 1989; Nobusawa *et al*, 1991; Lamb and Krug, 1996):

- binds infectious virus particles to sialic acid residues on the surface of susceptible host cells. The receptor binding site of the HA molecule is a pocket located in the distal tip of the spike (HA₁) and defined by a number of conserved amino acid residues (Tyr₉₈, Trp₁₅₃, His₁₈₃ and Leu₁₉₄);
- mediates the fusion (through the fusion peptide) of the endocytosed virus particle with the endosomal membrane, releasing the virus core into the cytoplasm. For this to occur the HA molecule has to be activated by proteolytic cleavage between HA₁ and HA₂ prior to anchorage of the protein to the host cell membrane;
- is the major antigen responsible for the generation of neutralising antibodies by the host. Changes in its antigenic structure are associated with recurrent influenza epidemics. There are 15 antigenic subtypes of HA infecting humans and animals: H1 to H15.

c) RNA segment 5 is 1565 nucleotides in length and encodes the 498 amino acid nucleocapsid protein (NP). The protein is rich in arginine residues but there are no clusters of basic residues as it might have been expected for interaction with the acidic

phosphate groups of RNA (Winter and Fields, 1981). Evidence points to the protein being phosphorylated at one serine residue per molecule (Kamata and Watanabe, 1997), although the percentage of molecules phosphorylated and its importance in the functionality of the protein is still unclear. NP is a type-specific antigen with a high degree of conservation amongst subtypes as confirmed by sequencing. Following synthesis in the cytoplasm, NP proteins have the ability of self targeting to the nucleus (Lin and Lai, 1983). Nuclear accumulation is achieved due to a group of a.a. contained within the NP protein (Davey *et al*, 1985), known as karyophilic sequence. The mechanisms of assembly of the nucleocapsid are not yet understood but it is known that once in the nucleus, NP associates with viral RNA forming a heterogeneous collection of structures which include free protein and ribonucleoproteins [RNP-RNA(+) and RNP-RNA(-)]. Due to the fact that NP does not associate with mRNAs it has been argued that a putative nucleation site is a stretch of 13 nucleotides conserved in the 5' end of all the RNA(-) and RNA(+) segments but absent from mRNAs (Hay *et al*, 1977).

d) RNA segment 6 codes for Neuraminidase (NA) or acylneuraminyl hydrolase. NA is composed of a single polypeptide chain. The spike of NA is a tetramer with four identical disulfide-linked subunits. It has a mushroom like structure composed of a "head" containing four planar and approximately spherical units attached to a central "stalk" which features a transmembrane hydrophobic region. NA contains five potential N-linked glycosylation sites and the cytoplasmic tail N-Met-Asn-Pro-Asn-Gly-Lys-C tends to be conserved amongst subtypes (Lamb and Krug, 1996). NA catalyses the cleavage of the α -ketosidic linkage between the terminal sialic acid and an adjacent sugar residue (normally D-galactose or D-galactosamine). This permits transport of the virus through mucin and prevents self-aggregation of the virus. Additionally, it destroys the HA receptor of the host cell, enabling elution of the progeny of virus particles from the infected cell. In short, NA confers mobility of the virus to and from the site of infection and replication (Laver *et al*, 1984). NA (like HA) is also a major subtype specific glycoprotein contributing to some of the antigenic variability revealed by influenza viruses. There are 9 distinct subtypes of NA, N1 to N9 (Ada and Jones, 1986).

e) RNA segment 7 encodes the matrix protein (M1) and the integral membrane protein (M2). M1 is the most abundant protein of the virion, it underlies the lipid envelop and confers rigidity to the membrane. Additionally it interacts with the cytoplasmic portions of HA, NA and M2 proteins and with the RNP structures. M1 is a type-specific antigen of influenza virus, highly conserved cross subtypes (Lamb, 1989). The M2 protein is predominantly expressed in the cytoplasmic membrane of infected cells, with only a few copies being incorporated by the virion. It is phosphorylated at Ser-64 and palmitylated at Cys-50. The active form of the protein is a homotetramer consisting of a pair of disulfide-linked dimers (Scholtissek, 1984). M2 functions as an ion channel that permits entrance of ions to the virion during uncoating, and modulates the pH of intracellular compartments, facilitating the budding process (Lamb and Krug, 1996).

f) RNA segment 8 encodes two proteins, non-structural proteins NS1 and NS2, present in influenza virus infected cells. NS1 protein binds specifically to poly(A) sequences, regulating nuclear export of mRNA, and to spliceosomes, inhibiting pre mRNA splicing. The NS2 protein, initially thought to be non structural, was recently identified in association with M1 protein underlining the virion envelope. Its function is not known (Lamb and Krug, 1996).

Viral replication

The interaction of cellular sialic acid residues with the distal portion of viral HA leads to adsorption of infectious virus to the target cell. The virus enters the cell by a process known as receptor-mediated endocytosis. After internalisation, endocytotic vesicles fuse to a cascade of endosomes of increasingly acidic pH that facilitate uncoating of the virion, followed by fusion of the viral membrane to the cellular membrane remnant, mediated by HA. This enables the RNPs to penetrate the cytoplasm and migrate to the nucleus, where they enter through the nuclear pore. In the nucleus viral RNA is transcribed to mRNAs and replicated in a process catalysed by the viral RNA polymerases. Newly assembled RNPs and mRNAs are exported from the nucleus to the cytoplasm where translation occurs. Viral integral membrane proteins (HA, NA and M2) are synthesised on membrane

bound ribosomes and translocated through the endoplasmic reticulum where carbohydrate chains are added. Once correctly folded and assembled, these proteins are further processed in the Golgi apparatus and finally expressed at the plasma membrane. Conversely, newly synthesised NP migrates back to the nucleus to associate with emerging viral RNAs. In polarised epithelial cells the virions assemble and bud at the apical surface of the cell. The precursor budding structure is a fragment of cell membrane with embedded viral envelope proteins. The M1 protein associates with the lipid bilayer and with RNP structures promoting interaction of the integral membrane proteins with the internal viral components. Release of fully formed virions from the plasma membrane is achieved by the destruction of cellular sialic acid receptors mediated by viral NA (reviewed in Lamb and Krug, 1996).

5.1.2 Immune response to influenza virus infection

The immune response to influenza virus infection involves nonspecific and specific components. Nonspecific mechanisms include fever, macrophages, natural killer (NK) cells, and interferon (Ada and Jones, 1986). The specific immune response mounted by hosts towards influenza virus infection is both humoral and cell mediated.

Humoral response

During infection with influenza A, antibodies to HA, NA, NP and M proteins are produced. Antibodies to HA and NA are normally associated with protection, whereas antibodies to NP and M1 are not. (Couch and Kasel, 1983). Neutralising antibodies to HA are easier to detect and thus more comprehensively studied.

Different types and forms of HA-specific neutralising antibodies are found in nasal and bronchial secretions and in the serum of hosts infected with influenza virus. Serum IgG and monomeric IgA and IgM subtypes can be detected after a primary infection, whereas IgG and IgA antibodies are predominant in secondary responses. In the case of mucosal antibodies, whilst oligomeric IgM, secretory IgA (sIgA) and, less frequently, IgG are

present in primary infection, after secondary infection, local antibody is mainly of the IgA isotype (Murphy and Clements, 1989; Ada and Jones, 1986).

Antibodies exert their antiviral activity either by mediating lysis of infected cells or by viral neutralisation. Lysis of infected cells is achieved by both the alternate pathway of complement and antibody-dependent cell-mediated cytotoxicity (ADCC) accomplished by NK cells and to lesser scale by neutrophils and monocytes (Ada and Jones, 1986). Neutralising IgM and sIgA prevent the attachment and/or internalisation of virus into the cells by sterical blockage of cell receptors. In contrast, IgG and monomeric IgA do not affect binding and internalisation of the virus, inhibiting viral replication through inactivation of the viral transcriptase (Possee *et al*, 1982; Taylor and Dimmock, 1985). The generation of anti-HA antibodies results in host protection against influenza infection, demonstrated by the correlation between antibody titres and resistance to infection. This resistance whilst very potent to homologous challenge is not cross protective to other subtypes of the virus. Antibodies also appear to have an additive but not essential role in the recovery from influenza infection (Ada and Jones, 1986).

Cell-mediated immune response

Influenza virus-specific cellular immune functions are mediated by two subsets of T lymphocytes. The first subset, cytotoxic T lymphocytes (CTLs) have a CD8⁺ phenotype and only recognise antigens processed by target cells in the context of the class I major histocompatibility complex (Male *et al*, 1996). Influenza antigens recognised by CTL clones include HA and the internal proteins NP, M and PB2. Recognition of HA accounts for the subtype specificity of some clones, whereas recognition of internal proteins and in particular NP, is responsible for cross-reactivity between subtypes of virus and for the generation of memory. (Yewdell and Hackett, 1989).

The second subset of T lymphocytes, belong to the T helper (Th) class of T cells, bearing CD4⁺ phenotype. These cells are MHC class II restricted and their functional properties are two fold: firstly, providing help for antibody production by B cells and for

proliferation of CD8⁺ T cells and secondly having cytolytic activity with similar specificity to that of the MHC I restricted T cells (Roitt *et al*, 1998). In addition, Th cells specific to NP can provide help to B cells secreting anti-HA antibody (Scherle and Gerhard, 1986).

The development of cell mediated responses has been proposed as the single most important host factor responsible for recovery from influenza virus infection (Ghendon *et al*, 1989). Indeed, the level of memory CTLs is associated with increased clearance of virus from the respiratory tract following secondary infection (Mumford *et al*, 1983).

Mechanism of immune evasion

A direct consequence of the selective pressure influenced by host immunocompetence is the frequent and progressive antigenic variation that influenza viruses undergo (Smith and Palese, 1989). These antigenic changes are achieved by the phenomena known as antigenic drift and antigenic shift and occur predominantly in the haemagglutinin and neuraminidase glycoproteins (reviewed in Webster *et al*, 1982; Russell and Edington, 1985; Both *et al*, 1983; Lamb and Krug, 1996).

Antigenic drift is caused by occasional mutations that determine accumulation of amino acid changes leading to minor differences in the antigenic character of the virus. If the mutations take place in epitopes recognised by neutralising antibodies they will be serologically detected by alterations in haemagglutination inhibition and neutralisation titres.

Antigenic shift is the main source of antigenic diversity of influenza virus and is characterised by extensive changes in the HA and/or NA proteins leading to the emergence of new subtypes of virus. There are three mechanisms thought to be involved, acting independently or in conjunction. The first is an increased mutation rate due to an augmented selective pressure. The second is the adaptation of virus from one species to another, chiefly from the avian pool to mammalian populations, supporting the concept

that avian populations serve as virus reservoir for other hosts. The third is a mechanism named genetic reassortment, which is the shuffling of RNA segments taking place between viruses of the same or even different host species and is a direct consequence of the segmented nature of the influenza virus genome.

5.1.3 Equine Influenza

History and evolution

Equine influenza is an acute respiratory disease of horses caused by infection with influenza virus type A. Only two antigenic subtypes have been isolated from horses to date: A/equine/1 virus of the H7N7 subtype and A/equine/2 virus of H3N8. The first virus of the H7N7 type was isolated during an outbreak in Czechoslovakia in 1956 and was named A/equine/Prague/1/56 (Sovinova *et al*, 1958). Based on genetic and antigenic analyses, type 1 viruses were divided into two subgroups with minor antigenic changes, due to antigenic drift. The first subgroup isolated between 1956 and 1963 (e.g. A/Cambridge/1/63) and the second, with isolations in 1964-77 (e.g. A/Detroit/1/67) (Gibson *et al*, 1992). Type 1 virus have not been detected since the late 1970's, although antibodies to this virus can still be detected in unvaccinated horses, supporting the idea that they continue to circulate in a subclinical form (Mumford, 1991).

During a major outbreak in the United States in 1963 the isolates were antigenically very distinct from the type 1 viruses, representing an antigenic shift, resulting in the substitution of both HA and NA glycoprotein antigens, probably due to reassortment with influenza virus of other species. They were designated A/equine/2 (H3N8) viruses and the first isolate was A/equine/Miami/1/63 (Wadell *et al*, 1963). Two antigenic variants have been recognised one having the Miami isolate as prototype and the second represented by the prototype variant A/Fontainbleu/79 (Kawaoka *et al*, 1989). Since 1987 these antigenic variants have further diverged, being currently classified in two distinct groups. The first circulating mainly in the Americas (American-like, e.g. A/Kentucky/1/94) and the other circulating in Europe and Asia (European-like, e.g. A/Newmarket/2/93). Nevertheless, there is evidence for significant intermingling in the distribution of these viruses. An

example, of American-like viruses isolated in Europe is the A/Newmarket/1/93 (Daly *et al*, 1996).

In 1989 a severe outbreak in northern China, with unusually high morbidity and mortality rates, led to the emergence of a new H3N8 virus very different from previous isolates. The isolate was named A/Jillin/89 (Guo *et al*, 1992) and phylogenetic analysis revealed it was closely related to avian viruses, suggesting it may have been directly transferred to horses from ducks without genetic reassortment (Guo *et al*, 1992). Although H3N8 viruses enjoy a significantly greater antigenic variability than those of the H7N7 subtype, they remain relatively similar to the prototype reference strains with homologies greater than 70% (Ada and Jones, 1986).

Epidemiology

Influenza viruses are a major cause of equine respiratory disease worldwide. The only significant equine populations in which influenza has not been reported are in Australia, New Zealand and Iceland. The disease is endemic in the U.S. and some areas of Europe with localised outbreaks every year and more extensive and severe outbreaks at intervals (Powell *et al*, 1991). Horses of all ages, not previously exposed or vaccinated, are susceptible. Infection, however, is more common in young (2 to 3 years) race and show horses due to stressful live style, frequent transport and concentration in confined spaces with horses of different provenience. Outbreaks commonly start when large numbers of susceptible horses are brought together at sales or shows (Ames, 1995). Infection may occur in vaccinated horses, with virus shedding but reduced or absent clinical signs, contributing significantly to the propagation of the disease (Mumford, 1991). Affected horses shed large amounts of aerosolised virus through respiratory secretions. The incubation period is short and shedding of virus persists for up to ten days (Ames, 1995).

Pathogenesis

Virus present in aerosols is inhaled and deposits on the mucosa of the upper and lower respiratory tracts. The mucus barrier is penetrated by the enzymatic activity of the viral NA. The virus attaches through the HA spikes to receptors on the epithelial cells, is endocytosed and released in the cell cytoplasm. The virus replicates in the host cells with budding of new virion particles that are spread throughout the respiratory tract (within 1 to 3 days) aided by ciliary action, infecting other cells or being expelled by cough. This process causes extensive epithelial damage leading to inflammation, clamping of cilia and focal erosions. Pulmonary alveolar macrophage function is compromised and secondary bacterial bronchopneumonia may result (Coggins *et al*, 1979; Ames, 1995).

Clinical signs

The onset of clinical manifestations is sudden with a rapid spread. The severity and duration of the signs is dependent on the immune status of the host, dose and virulence of the strain, and management and environmental conditions. Clinical findings include: harsh, dry and frequent cough; fever; lethargy; anorexia and serous bilateral nasal discharge, becoming mucopurulent in secondary bacterial complications (Powell, 1991; Ames, 1995). Partially immune or vaccinated horses usually have mild clinical signs or a subclinical process manifested only by intolerance to exercise.

Diagnosis

A presumptive diagnosis may be made on the basis of the clinical signs and rapid spread of the disease. Confirmation is achieved by virus isolation from nasopharyngeal swabs and demonstration of a significant rise in specific antibody titre in paired sera collections, 14 to 21 days apart (Powell, 1991). Methods of viral detection include culture of virus in embryonated specific pathogen free eggs and more recently in Madin-Darby canine kidney (MDCK) cell lines, antigen-capture indirect ELISA for the detection of the NP antigen and immunofluorescent staining of infected epithelial cells in air-dried nasal

smears (Mumford, 1991). More recently a method has been described for the detection of influenza virus infection by RT-PCR using internal primers for the segment 7, M1 gene (Donofrio *et al*, 1994). Serological tests, used in the diagnosis of influenza virus infection (Powell, 1991; Mumford, 1991) include the detection of neutralising antibodies against HA glycoprotein by haemagglutination inhibition (HI), single radial haemolysis (SRH) or virus neutralisation (VN), and the quantification of virus-specific IgM and IgG antibodies by radioisotopic anti-globulin binding assay (RABA). By 7 days after infection antibodies are detectable, peaking at about 3 weeks as assessed by HI, SRH and VN. Titres remain detectable for up to 18 months using RABA.

Treatment

Treatment is mainly symptomatic and comprises complete rest for a minimum of 3 to 4 weeks in a clean and well ventilated environment with minimum dust, antibiotics in significant secondary bacterial infections, non-steroidal anti-inflammatory drugs to treat fever, depression and muscle stiffness and bronchodilators and mucolytics to ease cough and improve breathing (Coggins, 1979; Powell, 1991; Ames, 1995). In uncomplicated cases the clinical signs improve within a week and most horses show complete clinical recovery in 2 to 3 weeks, although performance may be suboptimal for a prolonged period of time.

Prevention and control

Measures to be taken if a group of horses is to be maintained free of influenza involve quarantine of new arrivals for a minimum of 3 weeks, maintenance of adequate ventilation in the stables and routine vaccination (Ames, 1995). Current commercial vaccines (Glennon *et al*, 1996) contain both equine influenza subtypes as inactivated whole virus antigens added to adjuvants or presentation systems (Table 5.2). Adjuvants like polymers (e.g. Carbomer) or presentation systems composed by immune-stimulating complexes (ISCOMs) were included in vaccine formulations due to observations that they

significantly enhance and prolong the antibody response stimulated by inactivated viruses. The efficacy of vaccines against equine influenza virus is discussed in Chapter 6.

Equine influenza Haemagglutinin (HA) and Nucleoprotein (NP) were selected for use as immunogens in a DNA vaccination experiment in horses (Chapter 6). The choice of these two proteins is based on the fact that they stimulate different “arms” of the immune system of infected hosts. As previously discussed HA predominantly gives rise to the production of neutralizing antibodies whereas NP mainly stimulates cross-reactive cytotoxic T lymphocyte activity. Additionally the influenza strain Newmarket/2/93 was chosen to reflect circulating virus in Europe’s equine population. Below is a description of the strategy employed for the isolation and cloning of the HA and NP genes.

Table 5.2 Equine influenza vaccines currently available in the U. K.

Porduct Name	Marketing Company	Antigens Included ^a	Adjuvant	Dose	1 st Course	Immunization Schedule ^b Booster	Revaccination ^c
Prevac Plus	Hoechst Roussel Vet., Ltd	A/Equi-1/Prague/56 A/Equi-2/Miami/63 A/Equi-2/Suffolk/89	Carbomer	1 ml	2 doses, 4 to 6 weeks apart	2 doses, 6 months apart	1 dose annually
Equip F	Mallinckrodt Veterinary, Ltd	A/Equi-1/Newmarket/77 A/Equi-2/Brentwood A/Equi-2/Borlange/91	ISCOM ^d	2 ml	2 doses, 6 weeks apart	1 dose, 5 months after	1 dose annually
Duvaxyn IE Plus	Solvay Duphar Veterinary	A/Equi-1/Prague/56 A/Equi-2/Miami/63 A/Equi-2/Suffolk/89	Carbomer	1 ml	2 doses, 4 to 6 weeks apart	2 doses, 6 months apart	1 dose annually

^a All vaccines consist of whole inactivated viruses.
^b Administration by deep intramuscular injection.
^c Revaccinate more frequently (every 6 to 9 months) in high risk horses.
^d (ISCOM) Immunostimulating complexes.

5.2 Materials and Methods

5.2.1 Cloning of influenza virus genes

The genes encoding the influenza virus proteins HA and NP were recovered by RT-PCR of the viral RNA genome and cloned into a vector designed for the transfection of mammalian cells, promoting expression of the foreign genes of interest.

Extraction of Viral RNA

RNA from the virus strain A/Equine/Newmarket/2/93 was extracted from a 320X concentrated suspension of virus supplied by Intervet International B.V. (Boxmeer, The Netherlands) using the following protocol (modified from Sambrook *et al*, 1989).

In a Falcon 50 ml tube, 1 ml of virus suspension was added to 9 ml of Guanidinium Extraction Buffer (4M Guanidine Thiocyanate; 5mM Sodium citrate [pH 7.0]; 0.5% Sarkosyl [w/v]; DEPC treated dH₂O to 100 ml, adding 0.7% [v/v] β -mercaptoethanol immediately before use). The mixture was homogenised by passing through a 23-gauge needle not less than 10 times. To the homogenate was added in this order: 0.9 ml of 2M Sodium Acetate (pH 4), 9 ml of water saturated phenol (pH 8) and 2 ml of Chloroform: Isoamyl alcohol (24:1), after which it was shaken vigorously for 10 sec. and incubated on ice for 15 min. This was then centrifuged at 3,500 rpm (2,800 g) for 15 min at 4°C in a benchtop centrifuge (Beckman GS-6). After centrifugation the upper aqueous phase was harvested to a fresh tube and 10 ml of phenol: chloroform: isoamyl alcohol (25:24:1) was added. The mixture was once again shaken, incubated on ice and centrifuged as before. The aqueous phase was transferred to a Beckman centrifuge bottle (16x80mm) and precipitated by the addition of 1/10 volumes of 2M Sodium acetate and 2.5 volumes of ice cold 100% ethanol and freezing at -20°C for 1 hour. The RNA was pelleted by centrifugation at 10,000 rpm (7,800 g) for 20 min at 4°C in a Beckman J2-21centrifuge using a Beckman JA-20 rotor. The pellet was resuspended in 400 μ l of Guanidinium extraction buffer and transferred to an eppendorf tube. 50 μ l of 2M Sodium acetate and 1

ml of ice cold 100% ethanol was added and the tube was incubated at -20°C for 1 hour and centrifuged for 10 min in a microcentrifuge at maximum speed (13,000 rpm). The supernatant was discarded and the resulting pellet was washed with ice cold 70% ethanol and centrifuged for 5 min at 13,000 rpm. The pelleted RNA was then freeze-dried and resuspended in 50 µl of DEPC treated water. Upon completion of extraction 5 µl of RNA suspension was diluted to 1 ml with ddH₂O and its yield and purity assessed by spectrophotometry. N.B. To minimise RNA degradation, all the reagents were made up and all the tubes washed with DEPC treated water.

Primer design

Primer sequences were required for the amplification of the Newmarket/2/93 HA and NP genes. To that effect, known sequences of HA and NP from various Equine influenza H3N8 isolates were aligned using the Pileup program (UW-GCG software package), therefore enabling the design of primers from conserved regions upstream and downstream the open reading frames. In the case of the HA gene, primers (Table 5.3) were designed on the basis of the alignment (Figure 5.3) of the following list of sequences with respective accession numbers and citations: A/Equine/Kentucky/1/90-L39915 (Daly *et al*, 1995 unpublished); A/Equine/Newmarket/1/93-X85088 and Newmarket/2/93-X85089 (Daly *et al*, 1996 unpublished); France/1/76-M73773 (Bean *et al*, 1992); Santiago/1/85-M24725 and Algiers/72-M24721 (Kawaoka *et al*, 1989). As for NP, the primers shown in Table 5.4 were generated from aligned sequences as follows (figure 5.4): A/Equine/Kentucky/2/86-M30751 and Tennessee/2/86-M30758 (Gorman *et al*, 1990); Miami/1/63-M22575 (Gammel *et al*, 1989) and Jillin/1/89-M63786 (Gorman *et al*, 1991). The primers designed in this fashion were synthesised by a departmental contractor (MWG-Biotech, Germany). Melting temperatures were determined for the binding sequences of each primer, using the formula: $T_m = 4x(G+C) + 2x(A+T)$ (Itakura *et al*, 1984).

	1	FLU1 primer	HA-RT & HA1 primers	50
Kentucky90		AGCAAAAGCA GGGGATATTT	CTGTCAATCA TGAAGACAAC CATTATTTTG	
Newmarket293		-----	-----A TGAAGACAAC CATTATTTTG	
Newmarket193		-----	-----A TGAAGACAAC CATTATTTTG	
France76		AGCAAAAGCA GGGGATATTT	CTGTCAATCA TGAAGACAAC CATTATTTTG	
Santiago85		AGCAAAAGCA GGGGATATTT	CTGTCAATCA TGAAGACAAC CATTATTTTG	
Algiers72		AGCAAAAGCA GGGGATATTT	CTGTCAATCA TGAAGACAAC CATTATTTTG	

	1701	FLU2/HA2 primer	1762
Kentucky90	AACATCAGAT GCAACATTTG	CATTGAGTA AACTGATAGT TAAAAACACC	CTTGTTTCTA CT
Newmarket293	-----	-----	-----
Newmarket193	-----	-----	-----
France76	AACATCAGAT GCAACATTTG	CATTGAGTA AACTGATAGT TAAAAACACC	CTTGTTTCTA CT
Santiago85	AACATCAGAT GCAACATTTG	CATTGAGTA AACTGATAGT TAAAAACACC	CTTGTTTCTA CT
Algiers72	AACATCAGGT GCAACATTTG	CATTGAATA AACTAATAGT TAAAAACACC	CTTGTTTCTA CT

Figure 5.3 Fragment of HA sequence alignment showing conserved regions used for primer design.

Table 5.3 Primers for amplification of HA.

Primer	Sequence	Tm	Use
FLU1	5'- N NA CTAGTAGCAAAAGCAGGGGATATTTCTG-3'	66°C	cDNA /5' PCR
FLU2	5'- NNGCGGCCGCTTA ACT ATCAGTTTACTT-3'	46°C	3' PCR
HA-RT	5'- CTGTCAATCATGAAGACAACC -3'	60°C	cDNA Synthesis
HA1	5'- GGACGCGTCTGTCAATCATGAAGACAAC -3'	56°C	5' PCR
HA2	5'- ATGCGGCCGCTTA ACT ATCAGTTTACTC -3'	46°C	3' PCR

HA1 and HA2 primers contain 5' restriction enzyme sites, respectively MluI and NotI (underlined). Primer binding sequence is depicted in bold. These primers, used in conjunction, should produce a fragment of approximately 1740 bp, containing the HA coding region.

	1	NP-RT primer	NP1 primer	50
Kentucky86		AGCAAAAGCA GGGTAGATAA TCACTCACTG	AGTGACATCA AAGTCATGGC	
Tennessee86		AGCAAAAGCA GGGTAGATAA TCACTCACTG	AGTGACATCA AAGTCATGGC	
Miami63		AGCAAAAGCA GGGTAGATAA TCACTCACTG	AGTGACATCA AAGCCATGGC	
Jilllin89		AGCAAAAGCA GGGTAGATAA TCACTCACTG	AGTGACATCA ACATCATGGC	

	1501	NP2 primer	1565
Kentucky86	TCTTATTTCT TCGGAGACAA TGCTGAGGAG TTTGACAGTT	AAAGAAAAAT ACCCTTGTTT	CTACT
Tennessee86	TCTTATTTCT TCGGAGACAA TGCTGAGGAG TTTGACAGTT	AAAGAAAAAT ACCCTTGTTT	CTACT
Miami63	TCTTATTTCT TCGGAGACAA TGCAGAGGAG TATGACAATT	AAAGAAAAAT ACCCTTGTTT	CTACT
Jilllin89	TCTTATTTCT TCGGAGACAA TGCAGAGGAA TATGACAATT	AAAGAAAAAT ACCCTTGTTT	CTACT

Figure 5.4 Fraction of NP sequence alignment displaying conserved motifs used for primer design.

Table 5.4 Primers for the amplification of NP.

Primer	Sequence	Tm	Use
NP-RT	5'- AGCAAAAGCAGGGTAGATAATCAC -3'	68°C	cDNA Synthesis
NP1	5'- GTAAGCTTCCGCCATGTCACTGAGTGACATCA -3'	46°C	5' PCR
NP2	5'- NNGCGGCCGCGTAGAAACAAGGGTATTTTCT -3'	58°C	3' PCR

NP1 has a 5' HindIII site followed by a Kozak sequence element whereas NP2 contains a 5' NotI restriction site. Primer binding sequence is depicted in bold. NP1 and NP2 should generate a PCR product of apporximately 1570 bp.

cDNA synthesis

cDNA was synthesised using “First Strand cDNA Synthesis Kit” (Pharmacia) with Newmarket/2/93 RNA as template, primed with FLU1 or HA-RT and NP-RT primers respectively, following the manufacturer instructions (mat&meth). The cDNA was denatured at 94°C for 5 min and incubated on ice for 2 min immediately before being used as template for PCR amplification.

PCR amplification

PREPARATION OF PCR “MASTER MIXES”

Pfu DNA polymerase (Stratagene) proofreading enzyme was used for the amplification of HA and NP. The same “master mix” was used for the amplification of both genes due to the fact that the templates have the same origin and therefore equal purity, having additionally a very similar size, permitting optimisation factors such as concentration of dNTP’s, salts and enzyme to remain constant. The final composition of master mix per aliquot was as follows: 5 µl of 10 X Reaction buffer; 1µl of dNTP’s at 10 mM each (to obtain a final concentration of 200 µM each); 5 µl of DMSO and 26 ul of miliQ H₂O. Aliquots were stored at -20°C until needed.

REACTION CONDITIONS

To each aliquot of master mix was added 1 µl of each primer HA1 and HA2 or NP1 and NP2 (at a final concentration of 0.3 µM each) and 10 µl of cDNA template. A hot start was performed at 95°C for 5 min, followed by the addition of 1 µl of Pfu polymerase (2.5

U). Cycling was performed in a Perkin Elmer 9600 Thermal Cycler with the following parameters: 30 cycles of denaturation at 94°C for 45 sec, annealing at 62°C for 1 min (55°C for 1 min in the case of NP) and extension at 72°C for 5 min; followed by an extra extension step at 72°C for 7 min and by chilling at 4°C. Conditions detailed are the result of optimization leading to increased specificity and reproducibility of amplification.

Cloning of PCR products

PCR products were cloned into pCR-Script vector using pCR-Script Amp SK(+) Cloning Kit (Stratagene). This vector system is designed for the cloning of blunt-end fragments such as the ones generated by Pfu polymerase. Fragments are cloned into a predigested vector at the *SrfI* restriction site (5'- GCCC / GGGC -3'). The ligation reaction includes the enzyme *SrfI* that maintains a high concentration of digested vector therefore reducing dramatically the recircularization of nonrecombinant vector. This obviously requires that the fragment to be cloned is free of *SrfI* recognition sites. To further increase cloning efficiency the manufacturer recommends the purification of PCR products by selective precipitation, removing excess of primers.

PURIFICATION OF PCR PRODUCTS

To the PCR products, in a eppendorf tube, was added 1/10 volume of 10X STE buffer (1M NaCl; 200mM Tris-HCl [pH 7.5]; 100mM EDTA) and an equal volume of 4M ammonium acetate. To this was added 2.5 volumes of 100% (v/v) ethanol at room temperature and the tube was centrifuged in a microcentrifuge at 10,000 x g for 20 min at room temperature. The supernatant was discarded and the DNA pellet washed with 200 µl of 70% (v/v) ethanol and centrifuged at 10,000 x g for 10 min at room temperature. The ethanol was carefully removed and the pellet freeze-dried. The DNA was resuspended to the original volume (40 µl) with TE buffer (10mM Tris-HCl [pH 7.5]; 1mM EDTA) and stored at 4°C until needed.

INSERTION OF PCR PRODUCTS INTO PCR-SCRIPT

To a 0.5 ml microcentrifuge tube the following reagents were added in order: 1 µl of pCR-Script DNA (at 10 ng/µl); 1 µl of 10X reaction buffer; 0.5 µl of 10mM rATP; 200 ng of PCR products HA or NP (for a insert:vector ratio of approximately 40:1); 1 µl of SrfI (5 U/µl); 1 µl of T4 DNA ligase and dH₂O to a final volume of 10 µl. The reaction was gently mixed and incubated at room temperature for 1 hour. Following completion of incubation time the reaction was heated at 65°C for 10 min and stored on ice.

TRANSFORMATION OF BACTERIA AND SCREENING OF RECOMBINANTS

The *E. coli* strain Max Efficiency DH10B was transformed using a routine transformation protocol (mat&meth). Plasmid DNA was prepared from the bacterial cultures by the “STET method” (mat&meth). For the screening of HA clones the DNA was digested by EcoRV. Digestion of HA recombinant clones cuts DNA in two places (once in the vector and once in the HA gene releasing a band of approximately 650bp for genes cloned in sense orientation and a band of 1150bp for antisense. In the screening of NP recombinant clones was used XhoI. Similarly, digestion of NP positive clones produces a band of approximately 1300bp for sense orientation and a band of 300bp for antisense.

Sequencing of cloned PCR products

PREPARATION OF DNA

Four positive clones for either HA and NP were subcultured by dilution at 1/1000 in 20 ml of LB ampicillin (at 100 ug/ml) and shaken overnight at 37°C. Small scale DNA preparations of the subcultures were obtained using QIAprep Miniprep kit (Qiagen), observing manufacturers instructions (mat&meth). DNA was analysed by ethidium bromide stained agarose gel electrophoresis (mat&meth) and was quantified by spectrophotometry.

DESIGN OF SEQUENCING PRIMERS

As in the case of PCR primers, oligonucleotide sequences obtained from conserved regions of the genes were utilised for the synthesis of sequencing primers for both HA and NP (figure 5.5 and table 5.5).

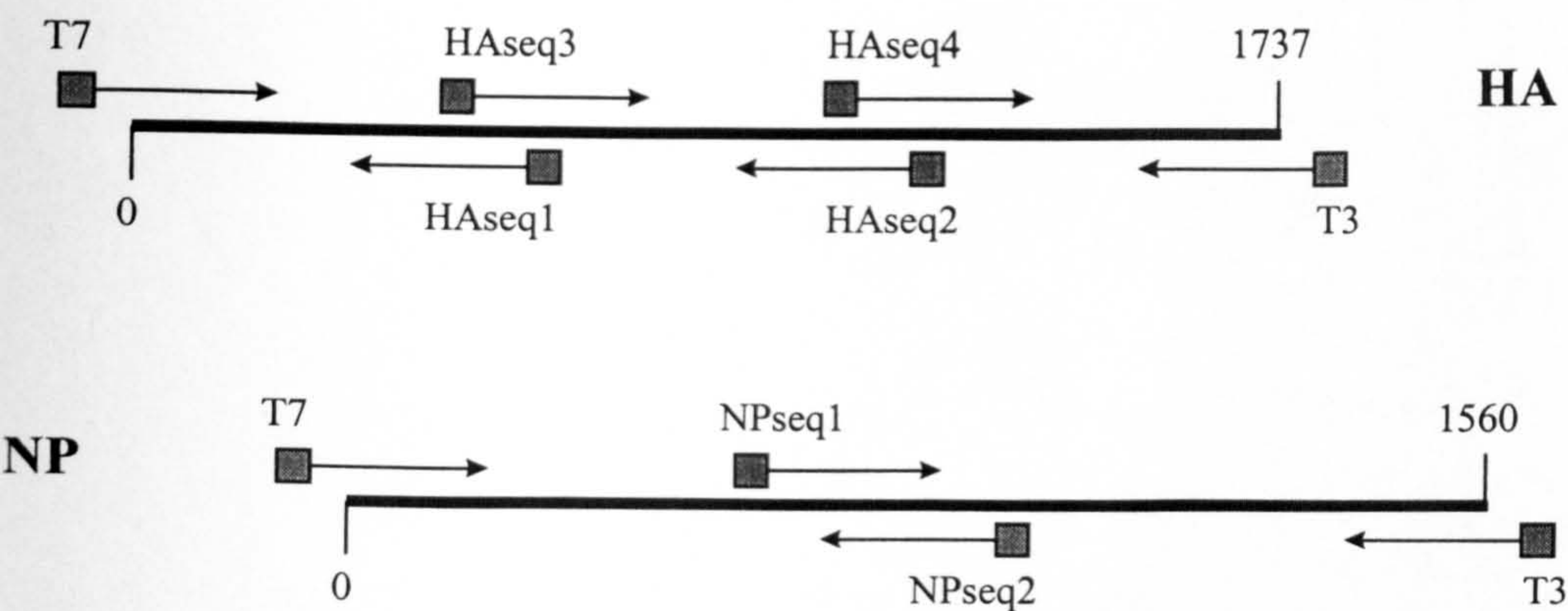


Figure 5.5 Schematic representation of HA and NP sequences showing sequencing primers.

Table 5.5 Sequencing primers for HA and NP.

Primer	Sequence	Tm	Target
T 3	5'- CAT TAA CCC TCA CTA AAG GG -3'	58°C	Vector
T 7	5'- TTA ATA CGA CTC ACT ATA GG -3'	54°C	Vector
HA seq1	5'- GTT TGA GCT CGG GTG ATG AAT C-3'	66°C	HA gene
HA seq2	5'- TTG ATG GAA TTT CTC ATT GG -3'	54°C	HA gene
HA seq3	5'- AAT TGG CTA ACA CAA TCT GG -3'	56°C	HA gene
HA seq4	5'- TGT GAT AAT GCA TGC AAT GG -3'	56°C	HA gene
NP Seq1	5'- CCA CAT ACC AAA GAA CAA GG -3'	58°C	NP gene
NP Seq2	5'- GTT CTG GAG TAG TTT GAA AGG -3'	60°C	NP gene

Care was taken to space the primers in sense and antisense orientation, taking in consideration that each run of sequencing generates between 500 and 700 bases of good quality sequence, as previous experience showed. All sequencing primers were synthesised by MWG-Biotech (Germany) and were modified by 5' labelling with the fluorochrome IRD-800.

SEQUENCING REACTIONS AND GEL RUN

Plasmid DNA templates and appropriate primers for HA and NP were mixed to produce sequencing reactions (mat& meth) using “ThermoSequenase Cycle Sequencing Kit” (Amersham). Cycle sequencing was performed in a Perkin Elmer 9600 thermal-cycler. Cycling conditions were: 94°C for 5 min followed by 25 cycles of denaturing at 98°C for 30 sec; annealing at 2°C below melting temperature of respective primer for 30 sec. and extension at 72°C for 40 sec; followed by chilling at 4°C. Sequencing reactions were run in a DNA automated sequencer (Li-Cor 4000L) using gel running conditions, solutions and buffers specified in the accompanying protocols (mat&meth).

DATA ANALYSIS

Sequence data generated from all the template : primer combinations, respectively for HA and NP, was analysed using UW-GCG software package. Consensus sequences for both HA and NP were produced using “Pileup” and “Pretty” programs. The consensus sequences were then translated using “Map” to derive the respective predicted amino acid sequences. The percentage of homology that the nucleotide and derived amino acid sequences exhibited towards other H3N8 influenza virus isolate sequences published or deposited in databases was determined using “Fasta” program.

5.2.2 Expression of influenza virus genes

Subcloning of HA and NP

The HA and NP genes were subcloned into pCI-Neo (Promega). As a preparatory step one clone from both HA and NP (showing 100% conservation relatively to the respective consensus sequences) was seeded at 1/1000 dilution in 20 ml of LB ampicilin (100ug/ml) and incubated overnight at 37°C with agitation. Two plasmid DNA minipreparations were performed for each using QIAprep Miniprep kit (Qiagen). Resulting DNA of each clone was pooled, analysed by ethidium bromide stained agarose gel and quantified by

spectrophotometry. Figures 5.6 and 5.7 show a graphical representation of the subcloning procedure.

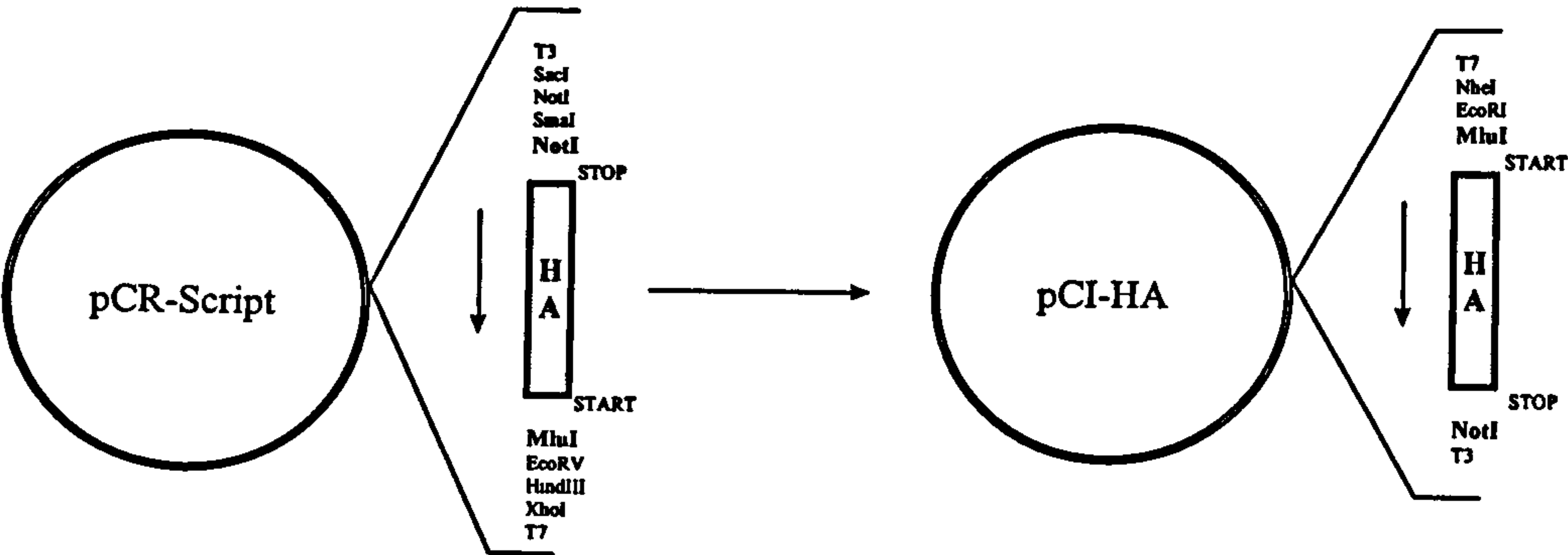


Figure 5.6 - Schematic of HA subcloning process. pCR-Script-HA recombinant DNA was cut with MluI and NotI. The resulting 1740 bp band was gel purified (mat&meth) and inserted into a MluI / NotI digested pCI-Neo.

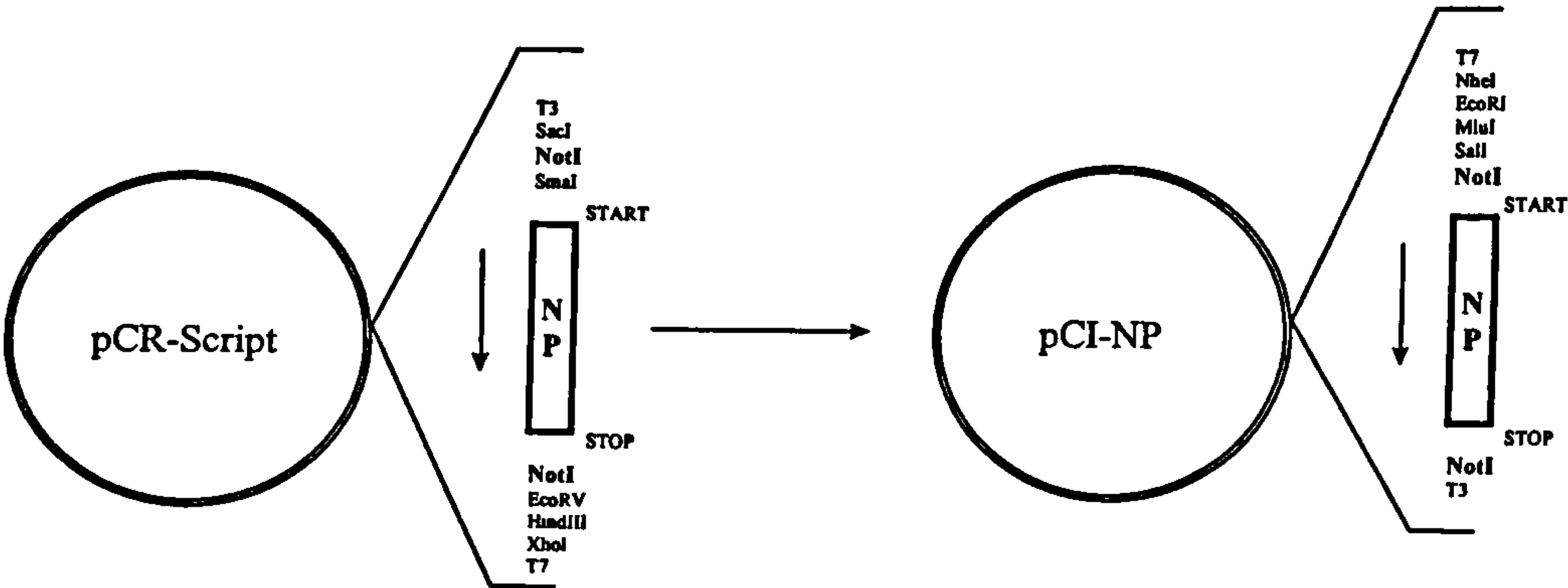


Figure 5.7 - Subcloning of NP. pCR-Script-NP was digested with NotI and the 1580bp product ligated into the NotI site of pCI-Neo

The bacterial cell strain Max Efficiency DH10B was transformed with both chimeras. In both cases, recombinant clones were screened with the restriction enzyme SstI with buffer with and BSA, producing the following band pattern: for HA 1000 bp for sense orientation and 1500 bp for antisense; for NP 900 bp for sense and 450 bp for antisense.

DNA from one clone (in sense orientation) for each chimera was large scale purified using, thoroughly checked by sequencing and taken through to the next stage.

Transfection of mammalian cells and RNA analysis

To investigate if the cloned genes were functional the highly transfectable COS-7 cell line was used for transient expression of HA and NP. Cells were transfected using the calcium-phosphate based “Mammalian Transfection Kit” (Stratagene) with the manufacturer guidelines. As a negative control cells were also transfected with empty pCI-Neo plasmid.

Transfected cells were collected by trypsinisation and centrifugation at different time points (24, 48 and 72 hours). Total RNA was extracted from cells using “Rneasy Midi Kit” (Qiagen). The RNA was quantified by spectrophotometry and analysed by northern blot (mat&meth) using gene specific radiolabelled probes. The probes, consisted of fragments of HA and NP, generated by restriction enzyme digestion of plasmid DNA and were labelled with [$\alpha^{32}\text{P}$]dCTP (Amersham) using “Oligolabelling Kit” (Pharmacia). A probe for the housekeeping enzyme GAP-DH was used as quality control of the RNA preparations.

5.3 Results

5.3.1 Amplification of HA and NP

Amplification of the HA gene was originally attempted using FLU1 primer for both cDNA first strand synthesis and upstream priming of the PCR and FLU2 primer for downstream priming. The PCR reaction was performed using Taq polymerase and resulted in a multiple band product (result not shown). This product was cloned and 3 of the clones corresponding to a full length HA gene were sequenced. These clones were later discarded due to the consistent presence of point mutations. Sequencing data derived from these clones was nonetheless useful in the design of improved gene specific primers (HA1 and HA2). In the case of NP, the primer NP-RT was used for cDNA first strand synthesis and primers NP1 and NP2 for 5' and 3' PCR priming, respectively. Amplification of the genes of interest was optimised through the titration of primer concentration, manipulation of cycling parameters and by the inclusion of DMSO in the PCR mix. Products of the expected size: approximately 1740 bp for HA and 1570 bp for NP were obtained (Figures 5.8 and 5.9). In both cases was included a negative control, consisting of dH₂O in place of cDNA template, to check for contamination of the reaction mix with foreign DNA.

5.3.2 Nucleotide and a.a. sequence of HA and NP

Nucleotide sequence of 4 HA clones and 4 NP clones was obtained using a series of gene specific and vector specific primers. Analysis of the resulting overlapping sequences was used to generate the consensus nucleotide and predicted amino acid sequences for HA and NP shown in figures 5.10 and 5.11, respectively.

5.3.2.1 Haemagglutinin

The sequence for nucleotides 1 through 27 was originally obtained from the cloned HA PCR product amplified using FLU 1 as a 5' primer, those clones were discarded as they

contained a point mutation (C substituted by T) in position 1120, which resulted in the introduction of a stop codon (TGA).

The open reading frame begins at the ATG codon (position 16) and ends at the TGA codon (position 1711), encoding a HA precursor of 565 amino acids. Newmarket/2/93 HA, shares between 98.1% and 99.4% nucleotide sequence and between 97.7% and 98.8% amino acid sequence identity with recent European and American isolates (Daly *et al*, 1995 and 1996 unpublished; Binns *et al*, 1993; Bean *et al*, 1992; Kawaoka *et al*, 1989). Further analysis of the amino acid sequence revealed conservation of the predicted N-terminal signal sequence cleavage site between positions 15 and 16 as well as the cleavage site that separates HA₁ from HA₂ that occurs between positions 344 and 345. Also conserved cross isolates are nine potential N-linked glycosylation sites, eight of them situated in the HA₁ portion of the molecule (at positions 18, 23, 37, 53, 68, 78, 180 and 300) and only one in the HA₂ portion in position 498 (reviewed in Daniels *et al*, 1985). Changes in the amino acid sequence across isolates take place predominantly in the HA₁ portion of the protein, reflecting selection pressure in the most antigenic portion of the molecule (Wiley *et al*, 1987).

5.3.2.2 Nucleoprotein

Analysis of the sequence revealed that the cloned NP product was amplified by primers NP2 and RT-NP (rather than the NP1 primer used in the PCR reaction). RT-NP primer carried over from first strand cDNA reaction had presumably a competitive binding advantage over NP1 primer. As described before the NP1 primer included a 5' tag containing HindIII and Kozak's sequence elements.

The predicted translation product of Newmarket/2/93 NP contains 498 amino acids, starting at codon ATG (position 46) and ending at codon TAA (position 1540). When compared with published NP sequences of different virus isolates (Gorman *et al*, 1990 and Gammelin *et al*, 1989), the nucleotide sequence and derived amino acid sequence of isolate Neumarket/2/93 exhibit an identity of 95.0-98.4% and 91.3%-93.4%, respectively.

The karyophilic sequence motif, responsible for nuclear accumulation of NP (Davey *et al*, 1985), which tends to be conserved among subtypes of human influenza A virus is present: 336-A-A-F/Y-E-D-L-R-V-L-344. Also a motif that resembles an epitope presented by infected cells to MHC class I- restricted CTL's (Gould *et al*, 1989) is present: 366- A-S-N-E-N-M-E-T-I-D-R-S-T-L-379.

5.3.3 Evidence of expression of HA and NP

Investigation of expression of HA and NP in a mammalian system, was only attempted by northern blot analysis. Figures 5.12-A and 5.13-A show auto-radiographs exposed to blots of RNA from cells transfected for 24, 48 and 72 hours, with plasmid encoding HA and NP respectively, labelled with gene specific probes. In each case is included a negative control consisting of cells transfected with empty expression vector (pCI-Neo). Figures 5.12-B/C and 5.13-B/C show the same blots labelled with a GAPDH probe, as a positive control for the RNA purification procedure.

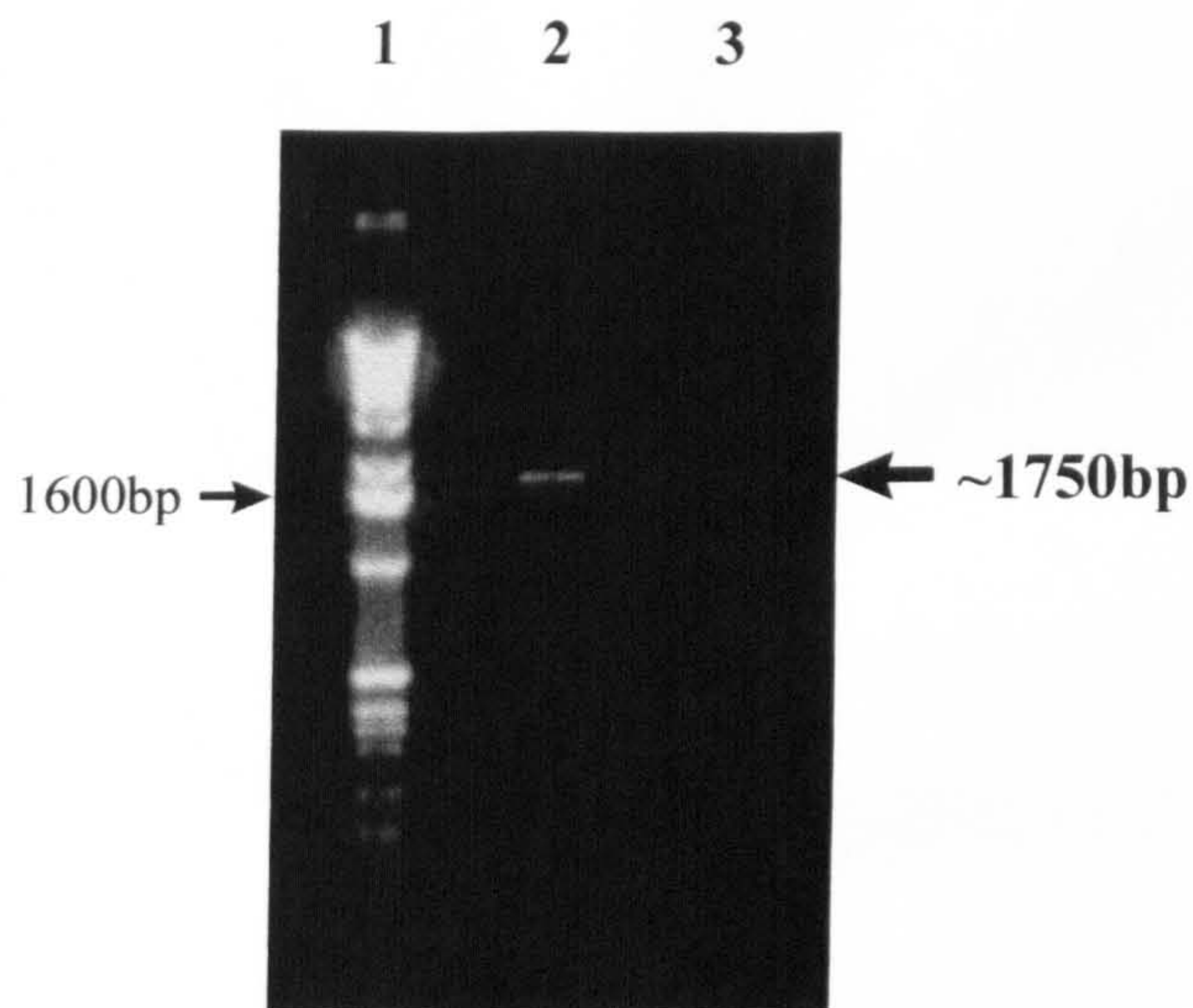


Figure 5.8 Haemagglutinin amplification products. Ethidium bromide stained agarose gel (1.2%). 10 μ l of each reaction loaded per lane. Lane1-1kb ladder; Lane2-HA product; Lane3- template control.

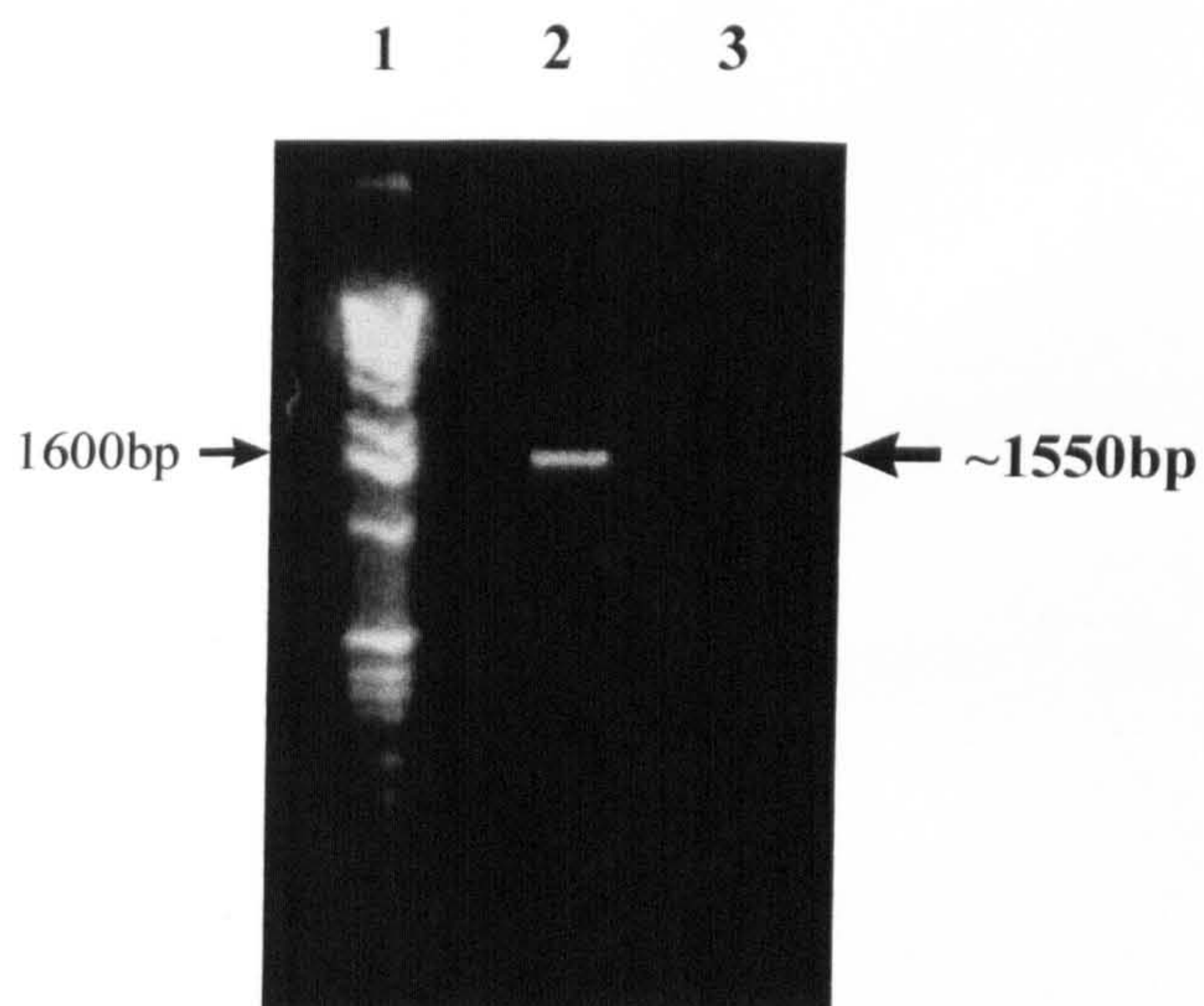


Figure 5.9 Nucleoprotein amplification products. Ethidium bromide stained agarose gel (1.2%). 10 μ l of each reaction loaded per lane. Lane1-1kb ladder; Lane2-NP product; Lane3- template control.

1 ACGCGTCTGTCAATCATGAAGACAACCATTATTTTGATACTACTGACCCATTGGGTCTACAGTCAAAACCCAACCAGTGGC 81
M K T T I I L I L L T H W V Y S Q N P T S G (22)

AACAACACAGCCACATTATGTCTGGGACACCATGCAGTAGCAAATGGAACATTGGTAAAAACAATAACTGATGACCAAATT 162
N N T A T L C L G H H A V A N G T L V K T I T D D Q I (49)

GAGGTGACAAATGCTACTGAATTAGTCCAGAGCATTTCATAGGGAAAATATGCAACAACTCATATAGGGTTCTAGATGGA 243
E V T N A T E L V Q S I S I G K I C N N S Y R V L D G (76)

AGAAATTGCACATTAATAGATGCAATGCTAGGAGACCCCCATTGTGATGATTTTCAGTATGAGAATTGGGACCTCTTCATA 324
R N C T L I D A M L G D P H C D D F Q Y E N W D L F I (103)

GAAAGAAGCAGCGCTTTCAGCAATTGCTACCCATATGACATCCCTGACTATGCATCGCTCCGGTCCATTGTAGCATCCTCA 405
E R S S A F S N C Y P Y D I P D Y A S L R S I V A S S (130)

GGAACATTAGAATTACAGCAGAGGGGTTACATGGACAGGTGTCACTCAAAACGGAAGAAGTGGAGCCTGCAAAGGGGA 486
G T L E F T A E G F T W T G V T Q N G R S G A C K R G (157)

TCAGCCGATAGTTTCTTTAGCCGACTGAATTGGCTAACACAATCTGGAAATTCTTACCCCATATTGAATGTGACAATGCCT 567
S A D S F F S R L N W L T Q S G N S Y P I L N V T M P (184)

AACAATAAAAATTTGATAAACTATACATCTGGGGGATTCATCACCCGAGCTCAAACAAAGAGCAGACAAAATTATATATC 648
N N K N F D K L Y I W G I H H P S S N K E Q T K L Y I (211)

CAAGAATCAGGACGAGTAACAGTCTCAACAGAAAGAAGTCAACAAACAGTAATCCCTAACATCGGATCTAGGCCGGGGGTC 729
Q E S G R V T V S T E R S Q Q T V I P N I G S R P G V (238)

AGGGGTCAATCAGGCAGGATAAGCATATACTGGACCATTTGTAAAACCTGGAGATATTCTAATGATAAACAGTAATGGCAAC 810
R G Q S G R I S I Y W T I V K P G D I L M I N S N G N (265)

TTAGTTGCACCGCGGGGATATTTTAAATTGAGAACAGGGAAAAGCTCTGTAATGAGATCAGATGCACTCATAGACACTTGT 891
L V A P R G Y F K L R T G K S S V M R S D A L I D T C (292)

GTGTCTGAATGTATTACACCAAATGGAAGCATCCCCAACGACAAACCATTTCAAAATGTGAACAAAATTACATATGGAAAA 972
V S E C I T P N G S I P N D K P F Q N V N K I T Y G K (319)

TGCCCCAAGTATATCAGGCAAAACACTTTAAAGCTGGCCACTGGGATGAGGAATGTACCAGAAAAGCAAATCAGAGGAATC 1053
C P K Y I R Q N T L K L A T G M R N V P E K Q I R G I (346)

TTTGGAGCAATAGCGGGATTATAGAAAACGGCTGGGAAGGAATGGTTGATGGGTGGTATGGATTCCGATATCAAAACTCG 1134
F G A I A G F I E N G W E G M V D G W Y G F R Y Q N S (373)

GAAGGAACAGGACAAGCTGCAGATCTAAAGAGCACTCAAGCAGCCATAGACCAGATCAATGGAAAATTAAACAGAGTGATT 1215
E G T G Q A A D L K S T Q A A I D Q I N G K L N R V I (400)

GAAAGGACCAATGAGAAATTCCATCAAATAGAAAAGGAATTCTCAGAAGTAGAAGGGAGAATCCAGGATTTGGAGAAGTAT 1296
E R T N E K F H Q I E K E F S E V E G R I Q D L E K Y (427)

GTAGAAGACACCAAAATAGACCTATGGTCCTACAATGCAGAATTGCTGGTGGCTCTAGAAAATCAACATACAATTGACTTA 1377
V E D T K I D L W S Y N A E L L V A L E N Q H T I D L (454)

ACAGATGCAGAAATGAATAAATTATTCGAGAAGACTAGGCGCCAGTTAAGAGAAAACGCGGAAGACATGGGAGGTGGATGT 1458
T D A E M N K L F E K T R R Q L R E N A E D M G G G C (481)

TTCAAGATTTACCACAAATGTGATAATGCATGCATTGGATCAATAAGAAATGGGACATATGACCATTACATATACAGAGAT 1539
F K I Y H K C D N A C I G S I R N G T Y D H Y I Y R D (508)

GAAGCATTAAACAACCGATTTCAAATCAAAGTGTGAGTTGAAATCAGGCTACAAAGATTGGATACTGTGGATTTTCATTC 1620
E A L N N R F Q I K S V E L K S G Y K D W I L W I S F (535)

GCCATATCATGCTTCTTAATTTGCGTTGTTCTATTGGGTTTCATTATGTGGGCTTGCCAAAAGGCAACATCAGATGCAAC 1701
A I S C F L I C V V L L G F I M W A C Q K G N I R C N (562)

ATTTGCATTTGAGTAAACTGATAGTTAAGCGGCCGC 1737
I C I * (565)

Figure 5.10 - Nucleotide and predicted amino acid sequences of the HA gene for the A/Equine/Newmarket/2/93 (H3N8) influenza virus isolate. Nucleotides are numbered in bold and amino acids in brackets. The symbol * identifies the stop codon. Primers used for PCR amplification are underlined.

1 AGCAAAAGCAGG**TAGATAATCACTCACTGAGTGACATCAAAGTCATGGCGTCTCAAGGCACCAAACGATCTTATGAGCAG** **81**
M A S Q G T K R S Y E Q (12)

ATGGAAACTGATGGGGAACGCCAGAATGCAACTGAAATCAGAGCATCTGTCCGAAGGATGGTGGGAGGAATCGGCCGGTTT **162**
M E T D G E R Q N A T E I R A S V G R M V G G I G R F (39)

TATGTTTCAGATGTGTACTGAGCTTAAATTAAACGACCATGAAGGGCGGCTGATTCAGAACAGCATAACAATAGAAAGGATG **243**
Y V Q M C T E L K L N D H E G R L I Q N S I T I E R M (66)

GTACTTTTCGGCATTTCGACGAAAGAAGAAACAAGTACCTCGAGGAGCATCCAGTGCTGGGAAAGACCCTAAGAAAACGGGA **324**
V L S A F D E R R N K Y L E E H P S A G K D P K K T G (93)

GGCCCGATATACAGAAGGAAAGATGGGAAATGGATAAGAGAACTCATCCTCCATGATAAAGAGGAAATCATGAGGGTCTGG **405**
G P I Y R R K D G K W I R E L I L H D K E E I M R V W (120)

CGTCAGGCCAACAAATGGTGAAGATGCTACTGCTGGTCTTACCCATATGATGATCTGGCACTCCAATCTCAATGACACCACA **486**
R Q A N N G E D A T A G L T H M M I W H S N L N D T T (147)

TACCAAAGAACAAGGGCTCTTGTTCCGACTGGGATGGATCCCAGAATGTGCTCTCTGATGCAAGGATCAACCCTCCCACGG **567**
Y Q R T R A L V R T G M D P R M C S L M Q G S T L P R (174)

AGATCTGGAGCTGCCGGTGCTGCAGTAAAAGGTGTTGGAACAATGATAATGGAATCATCAGGATGATCAAACGCGGGATA **648**
R S G A A G A A V K G V G T M I M E L I R M I K R G I (201)

AATGATCGAAATTTCTGGAGAGGTGAAAaTGGTCGAAGAACCAGAATTGCTTATGAAAGAATGTGCAATATCCTCAAAGGG **729**
N D R N F W R G E N G R R T R I A Y E R M C N I L K G (228)

AAATTTCAAACAGCAGCACAAACGGGCTATGATGGACCAAGTGAGGGAGGGCCGCAATCCTGGAAATGCTGAGATTGAGGAT **810**
K F Q T A A Q R A M M D Q V R E G R N P G N A E I E D (255)

CTCATTTTCTTGGCACGATCAGCACTCATTTGAGAGGATCAGTAGCCCATAAATCATGCCTACCTGCCTGTGTTTATGGCT **891**
L I F L A R S A L I L R G S V A H K S C L P A C V Y G (282)

CTTGCACTAACCAGTGGGTATGACTTTGAGAAGGAAGGATACTCTCTGGTTGGGATTGATCCTTTCAAACACTACTCCAGAAC **972**
L A V T S G Y D F E K E G Y S L V G I D P F K L L Q N (309)

AGCCAAATTTTCAGTCTAATCAGACCGAAAGAAAATCCAGCACACAAGAGCCAGTTGGTGTGGATGgCATGCCATTCTGCA **1053**
S Q I F S L I R P K E N P A H K S Q L V W M A C H S A (336)

GCATTTGAGGACCTGAGAGTTTTGAATTTTATTAGAGGAACAAAAGTAATCCCAAGAGGACAGTTGGCAACCAGAGGAGTG **1134**
A F E D L R V L N F I R G T K V I P R G Q L A T R G V (363)

CAGATTGCTTCAAATGAAAACATGGAGACAATAGATTCTAGCACACTCGAACTGAGAAGCAGATATTGGGCAATAAGGACC **1215**
Q I A S N E N M E T I D S S T L E L R S R Y W A I R T (390)

AGAAGTGGAGGAAACACCAGTCAACAGAGAGCATCTGCAGGACAGATAAGTGTGCAACCTACTTTCTCAGTGCAGAGAAAT **1296**
R S G G N T S Q Q R A S A G Q I S V Q P T F S V Q R N (417)

CTTCCCTTTGAAAGAGCAACCATTATGGCTGCATTTACTGGGAACACTGAGGGGAGGACTTCCGACATGAGAACGGAAATC **1377**
L P F E R A T I M A A F T G N T E G R T S D M R T E I (444)

ATAAGGATGATGGAAATGCCAAATCAGAAGATGTGTCTTTCCAGGGGCGGGGAGTCTTCGAGCTCTCGGACGAAAAGGCA **1458**
I R M M E N A K S E D V S F Q G R G V F E L S D E K A (471)

ACGAACCcGATCGTGCCTTCCTTTGACATGAGCAATGAAGGGTCTTATTTCTTCGGAGACAATGCTGAGGAGTTTGACAGT **1539**
T N P I V P S F D M S N E G S Y F F G D N A E E F D S (498)

TAAAGAAAAATACCCTTGTTTCTACGCGGCCGC **1572**

★

Figure 5.11 - Nucleotide and predicted amino acid sequences of the NP gene for the A/Equine/Newmarket/2/93 (H3N8) influenza virus isolate. Nucleotides are numbered in bold and amino acids in brackets. The symbol ★ identifies the stop codon. Primers used for PCR amplification are underlined.

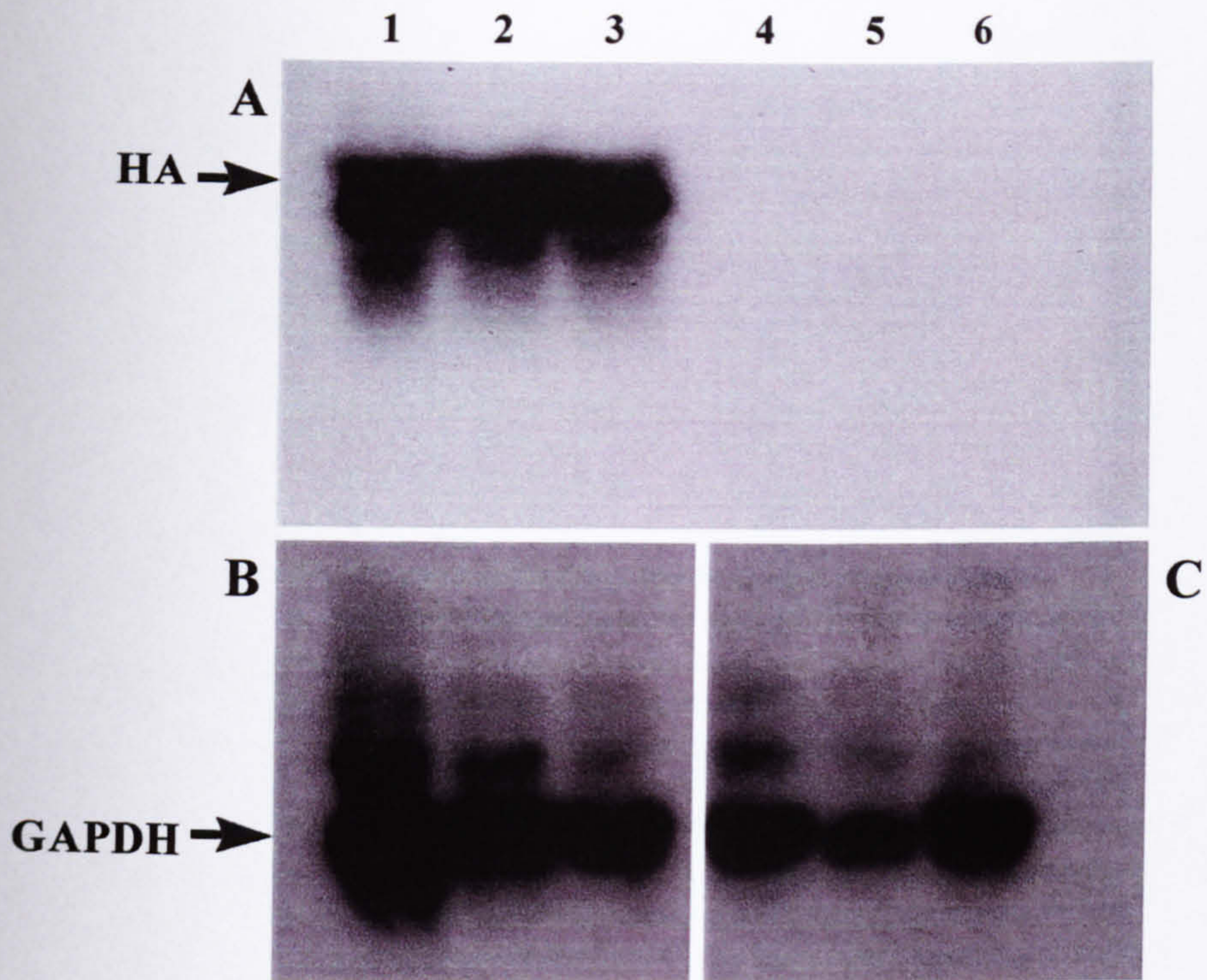


Figure 5.12 Expression of haemagglutinin in mammalian cells.

A- RNA derived from transfected COS-7 cells was probed with a HA gene specific probe. RNA (15 μ g/lane) was loaded as follows: Lanes 1, 2,3 pCI-HA at 24, 48 and 72 hours post-transfection, respectively; Lanes 4, 5, 6 pCI-neo parental plasmid at 24, 48 and 72 hours post-transfection, respectively.

B and C- The same samples as in gel A, loaded in the same order, were probed with a GAPDH probe.

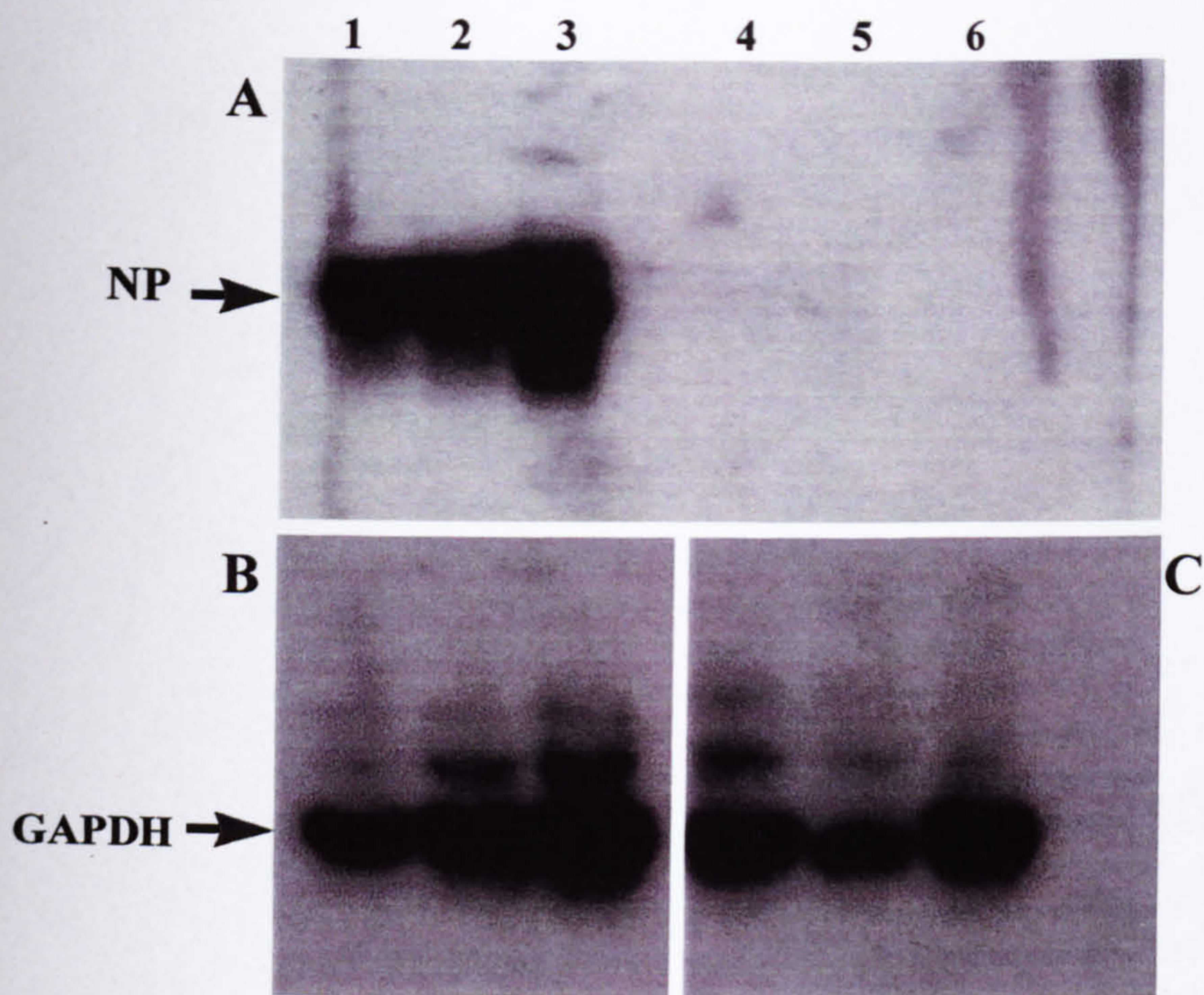


Figure 5.13 Expression of nucleoprotein in mammalian cells.

A- RNA derived from transfected COS-7 cells was probed with a NP gene specific probe. RNA (15 μ g/lane) was loaded as follows: Lanes 1, 2,3 pCI-NP at 24, 48 and 72 hours post-transfection, respectively; Lanes 4, 5, 6 pCI-neo parental plasmid at 24, 48 and 72 hours post-transfection, respectively.

B and C- The same samples as in gel A, loaded in the same order, were probed with a GAPDH probe.

5.4 Discussion

The purpose of this part of the project was to clone genes of equine influenza virus that could be used as a source of antigen in a DNA vaccination trial. The antigens selected for this experiment were the haemagglutinin representing the external and highly variable portion of the virus particle and the nucleoprotein representing the internal and conserved region of the virion. A discussion on the importance of these antigens in the elicitation of immune responses against influenza virus, particularly following DNA vaccination is presented in chapter 6.

The genes amplified and cloned show a high degree homology with corresponding sequences of other isolates both at the nucleotide and at the amino acid level. Furthermore there is conservation of functionally important features such as the proteolytic cleavage sites in HA and kariophilic sequence in NP. These observations attest for the authenticity of the cloned genes.

The HA and NP genes were subcloned into pCI-Neo and the capacity of the vector in driving their expression following transfection of COS7 cells was assessed by northern blot. The identity of the expressed proteins was later confirmed elsewhere by indirect fluorescent labeling of transfected cells using a serum of influenza positive horses as the primary antibody (Dr. Paul Sondermeijer, personal communication).

The cloning and expression of HA and NP will permit the assessment of their role in eliciting protection against influenza virus

CHAPTER 6

DNA VACCINATION AGAINST EQUINE INFLUENZA VIRUS

6.1 Introduction

6.1.1 DNA vaccines

Recent developments in the understanding of antigen recognition by T cells have established that an efficient immune response to most intracellular pathogens (especially viruses) is mounted only if CD8⁺ precursor cells are activated to generate MHC class I restricted cytolytic T cells (CTLs), along with an effective humoral response, in the shape of neutralising antibodies (Hassett and Whitton, 1996). Elicitation of humoral responses is easily achieved by using inactivated pathogens or subunit vaccines (e.g. recombinant proteins or synthetic peptides representing antigenic determinants) that can be directly recognised by B cells and processed by APCs for presentation to CD4⁺ T cells, providing help and memory for antibody formation (reviewed in Ahmed and Gray, 1996). In contrast, particularities in the processing of antigens for MHC class I presentation, suggest that polypeptides are efficiently presented only when expressed *de novo* in infected cells (Braciale, 1993). Until recently methods to promote *in vivo* expression of antigens had to rely on the infection of hosts with live (or attenuated) pathogens or replicating viral vectors encoding the gene of interest, with all the safety implications that they entail.

In 1990, Wolf *et al*, reported that plasmid DNA encoding the gene for a marker protein injected in striated muscle resulted in persistent expression of the gene at the site of injection. This observation led to the development of a new immunisation strategy named DNA vaccination (also known as genetic or polynucleotide immunisation) that consists of the inoculation of hosts with plasmid DNA, under the control of an appropriate promoter, encoding individual or collections of antigens, leading to the induction of specific immune responses to the antigens uptaken and expressed *in vivo* (Cohen *et al*, 1998).

Although most of the evidence on efficacy of this method of immunisation has been derived from studies in mice, increasing reports demonstrate its potential for vaccination of larger species including ferrets (Donnelly *et al*, 1995), pigs (Macklin *et*

al, 1998), cattle (Schrijver *et al*, 1997), dogs and cats (Osorio *et al*, 1999) and non-human primates (McCluskie *et al*, 1999).

The advantages presented by this method of immunisation (Robinson and Torres, 1997; Montgomery *et al*, 1997) besides its low cost are:

- the ease of production, as gene inserts can be recovered by PCR eliminating the need for production and purification of recombinant proteins and even the culture of the pathogen if PCR is performed using tissue extracts from infected hosts;
- the safety, as the plasmid will contain only selected antigenic determinants of the pathogen, practically abolishing the risk of pathogenicity and also the potential risk of genome integration associated with replicating live vectors;
- the simplicity of manipulating constructs using standard molecular biology techniques enables the investigation of alternative vaccination conditions such as the effect of the antigen form (secreted or bound to membranes), by inclusion of targeting signals in the construct, or the effect of co-expressed cytokines, by either engineering cytokine genes into the construct or co-administrating plasmids encoding cytokines;
- the fidelity of the expressed proteins, at both structural and conformational levels, ensures that the immune responses will be comparable to those elicited by the wild type pathogens, increasing therefore the likelihood of protection following challenge;
- the efficacy, as DNA vaccinations have been shown to be useful in immunisation against bacteria, virus, and parasites in animal models, giving rise to long lived cell mediated and/or humoral immune responses;
- the stability of DNA over temperatures ranging from sub zero to just under boiling.

The versatility of this technique is further emphasised by its use as a powerful tool for expressing antigens *in vivo* that overcomes the need for the production and purification of large quantities of recombinant protein to serve as inoculum in such instances as the production of monoclonal and polyclonal antibodies (Barry *et al*,

1994) and in the screening of complex mixtures of antigens for immunogenic potential, as is the case in the definition of protective antigens in diseases for which little is known about the mechanisms of protection (Barry *et al*, 1995).

Vectors for DNA vaccination

Plasmids for DNA vaccination (figure 6.1) are commercially available and were initially developed for recombinant protein expression by mammalian cell transfection *in vitro*, sharing features (reviewed in Montgomery *et al*, 1997; Robinson and Torres, 1997; Tighe *et al*, 1998) that include:

- a backbone consisting of an origin of replication (*ori*) for high copy number production in *E. coli* and an antibiotic resistance gene (e.g. ampicillin) for selection also in *E. coli*. Because the bacterial origin of replication is not functional in mammalian cells the plasmid does not replicate when injected into hosts, therefore greatly reducing the risk of genome integration;

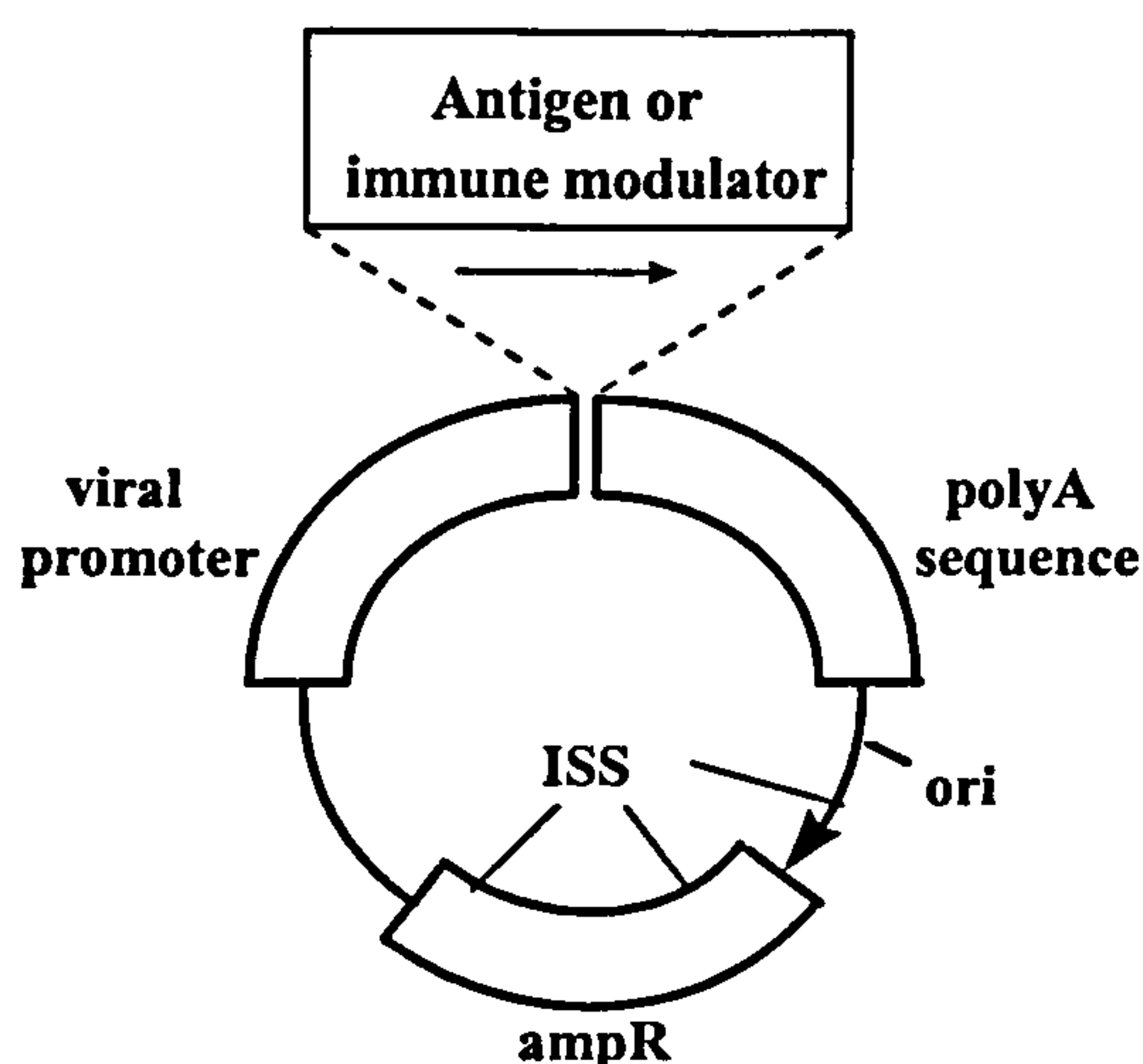


Figure 6.1 Features of plasmid suitable for DNA immunisation (modified from Robinson and Torres, 1997; Tighe *et al*, 1998).

- a promoter capable of directing high levels of gene expression in mammalian cells, normally strong viral promoters, including the human cytomegalovirus immediate early promoter (CMV-IE) and the Rous sarcoma virus long

terminal repeat (RSV-LTR). Some plasmids also include an intron and splicing elements as this was found to increase gene expression. A strong promoter is required as small amounts of plasmid are available for expression due to the low efficiency of plasmid uptake *in vivo* and to the absence of replication in mammalian cells;

- a 3'-untranslated region from genes of mammalian (e.g. bovine growth hormone) or viral (e.g. SV40) origin, for efficient transcription termination and polyadenylation (polyA).
- because of its bacterial origin, plasmid DNA contains immunostimulatory sequences (ISS) with a consensus sequence consisting of two purines (G/A), an unmethylated CpG motif and two pyrimidines (C/T). These sequences are rare in eukaryotic DNA and are therefore perceived as danger signals by the host immune system, evoking powerful cell mediated responses (Pisetski, 1997; Tighe *et al*, 1998). Most plasmids used for DNA immunisation have at least two of these sequences in the ampicillin resistance gene (ampR).

Antigens

Sufficient knowledge of the biology of natural infection is required for choosing a correct antigen (or antigens) that will elicit an appropriate immune response, not only to achieve protection, but also to prevent the development of immunopathology. Following choice of antigens and insertion of the appropriate genes into the plasmid vector it is important to assess expression of the protein of interest. This is conveniently done by transient transfection *in vitro* of a suitable cell line followed by measurement of RNA and/or protein expression (Ulmer *et al*, 1996). This might reveal the need for inclusion of post-transcriptional or post-translational modification signals such as an optimal sequence of translation initiation (Kozak sequence) for maximal expression, or a signal sequence for efficient trafficking or secretion of the protein (reviewed in Montgomery *et al*, 1997).

Administration of DNA vaccines

When deciding on a vaccination protocol, parameters such as route of administration, dose of plasmid DNA and number and interval between immunisations should be addressed.

DNA vaccines have been administered by a variety of routes either injected (e.g. intramuscular, intradermal, intravenous, intraperitoneal, subcutaneous) or non injected (e.g. intranasal, ocular, oral by feeding) and by using special apparatus (gene gun). Most studies have however been designed around the intramuscular or intradermal routes, because of early reports that these routes induce potent and long lived immune responses (reviewed in Fynan *et al*, 1993; McCluskie *et al*, 1999).

Intramuscular inoculation is generally performed by needle injection of plasmid DNA in saline. Striated muscle is 100 to 1000 times more efficient in uptake of injected DNA than other tissues (Wolf *et al*, 1990). This is in part due to the regeneration process of the injured muscle following injection as delivery of DNA to muscle previously damaged (e.g. by toxins or bupivacaine) results in an increased number of cells expressing the antigen and enhanced immune responses (Hassett and Whitton, 1996). The role of muscle fibres in antigen presentation is still unclear. Reports that transplantation of myoblasts stably transfected with antigen can induce both antibody and CTL responses and the fact that myoblasts can be activated (e.g. by IFN- γ) to express MHC class II molecules make a case for the function of muscle cells as APCs. On the other hand observations that surgical ablation of the inoculated muscle as soon as 1 minute post-inoculation has no effect on the development of immune responses and that muscle fibres are not able to express costimulatory molecules (e.g. B-7), suggests that the immune responses are mediated by professional APCs recruited to the injured muscle or, alternatively, by direct transfection of APCs at the injection site or by plasmid carried through blood to distal lymphoid tissues (reviewed in Hohlfield and Engel, 1994; Robinson, 1997; Cohen *et al*, 1998).

Although less efficient than the muscle in uptake of DNA, the skin constitutes, along with mucous membranes, the site where most antigens are first encountered and is

therefore specialised in supporting the initiation of immune responses. The skin immune system (SIS) is composed of several exclusive effector cells including keratinocytes that produce a number of cytokines (e.g. IL-1, IL-3, IL-6, IL-18, IFN- α/β , GM-CSF, TNF- α , TGF- β) upon non-specific stimulation, T cells, both infiltrating and resident (expressing cutaneous lymphocyte associated antigen) and Langerhans cells, constituting around 8% of the live cells of the epidermis, that differentiate into dendritic cells (professional APCs). An additional feature of the skin is the dermal perivascular units, structures with high concentration of immune cells (mostly T cells) in close association with blood vessels. These characteristics lead to the notion that the skin is a unique immunological microenvironment able to quickly and efficiently direct both innate and acquired immune responses (reviewed in Bos and Kapsenberg, 1993; Salmon *et al*, 1994). Plasmid DNA can be transfected intradermally in two forms. In a saline solution either by needle injection or by needlesh inoculation using a device driven by compressed gas to deliver a thin stream of inoculum under high pressure, capable of penetrating tissue. Alternatively DNA can be coated onto gold particles and accelerated into the epidermis using an apparatus named gene gun, which uses chemical expansion of water vapour or compressed gas as propellant (reviewed in Donnelly¹ *et al*, 1997).

The comparison of several routes and methods of delivery has established that efficiency of transfection does not always correlate with efficacy in the development of immune responses. For instance the greater ability of muscle to uptake DNA was not associated with an enhanced efficacy of vaccination (Fynan *et al*, 1993). In contrast, gene gun mediated bombardment is, by far, the most efficient method of immunisation eliciting similar levels of CTL and antibodies with 100 to 5000-fold less DNA than required by injection. An amount of DNA as little as 16ng (in mice) or 200ng (in larger animals) delivered by gene gun induces immune responses only obtainable with the inoculation of 10 and 100 μ g (respectively in mice and larger animals) by intramuscular or intradermal injection (Montgomery *et al*, 1997, Cohen *et al*, 1998). These studies also showed that the way in which DNA is delivered has a bearing on the type of immune response elicited. Intramuscular injection produces predominantly a Th1 type response whereas repeated gene gun inoculation culminates

in the induction of a Th2 type response. Intradermal injection appears to be able to induce both Th1 and Th2 responses (Robinson, 1997; Cohen *et al*, 1998).

The number and frequency of immunisations is interrelated and dependent on the efficacy of the route adopted. While large numbers of inoculations (4 or more) are generally given at short time intervals (e.g. 4 weeks), smaller numbers of inoculations (2 or 3) will correspond to larger intervals (8 to 12 weeks). A longer rest period between immunisations has been found to enhance immune responses (Prayaga *et al*, 1997; McCluskie *et al*, 1999).

Due to similarities between the immune systems and pathways of antigen presentation of mammals some extrapolations for vaccination protocols can be made from murine model studies (Robinson and Torres, 1997). Nevertheless, results in mice were found to be not always predictive to those in higher mammalian species [e.g. monkey (McCluskie *et al*, 1999); dogs and cats (Osorio *et al*, 1999)]. Therefore, optimal dose, route and immunisation schedule should ideally be assessed for each antigen/host system to be used.

Mechanism of action and immune responses

Delivery of DNA results in the transfection of cells in the target tissue. Upon entry in the cells, plasmid DNA is transported to the nucleus where it directs expression of the inserted gene. The protein antigen expressed in this fashion is acted upon by cells of the immune system, following membrane presentation or secretion by the transfected cell. It has been calculated that an efficient immune response can be initiated by the transfection of a single APC with approximately 100 molecules of plasmid DNA out of the 10^{10} molecules delivered by injection of 100 μ g of DNA or the 10^8 gold/DNA complexes delivered by gene gun (Robinson and Torres, 1997).

In the skin, the amount of tissue specific dendritic cells (Langerhans cells) and other immune competent cells (discussed above) is consistent with high efficiency in the initiation of immune responses following DNA delivery. In the muscle it is more

difficult to define the role of dendritic cells (due to their small numbers) in uptake of DNA for de novo expression of protein required for MHC I presentation. It was nevertheless shown that following DNA delivery APCs do express MHC class I molecules (Doe *et al*, 1996). It is also difficult to concede that muscle fibres are able to professionally present MHC class II molecules. It is probable that both cell types play a role in the initiation of immune responses following *in vivo* transfection of DNA. One hypothesis that attempts to reconcile this dispute is presented in figure 6.2.

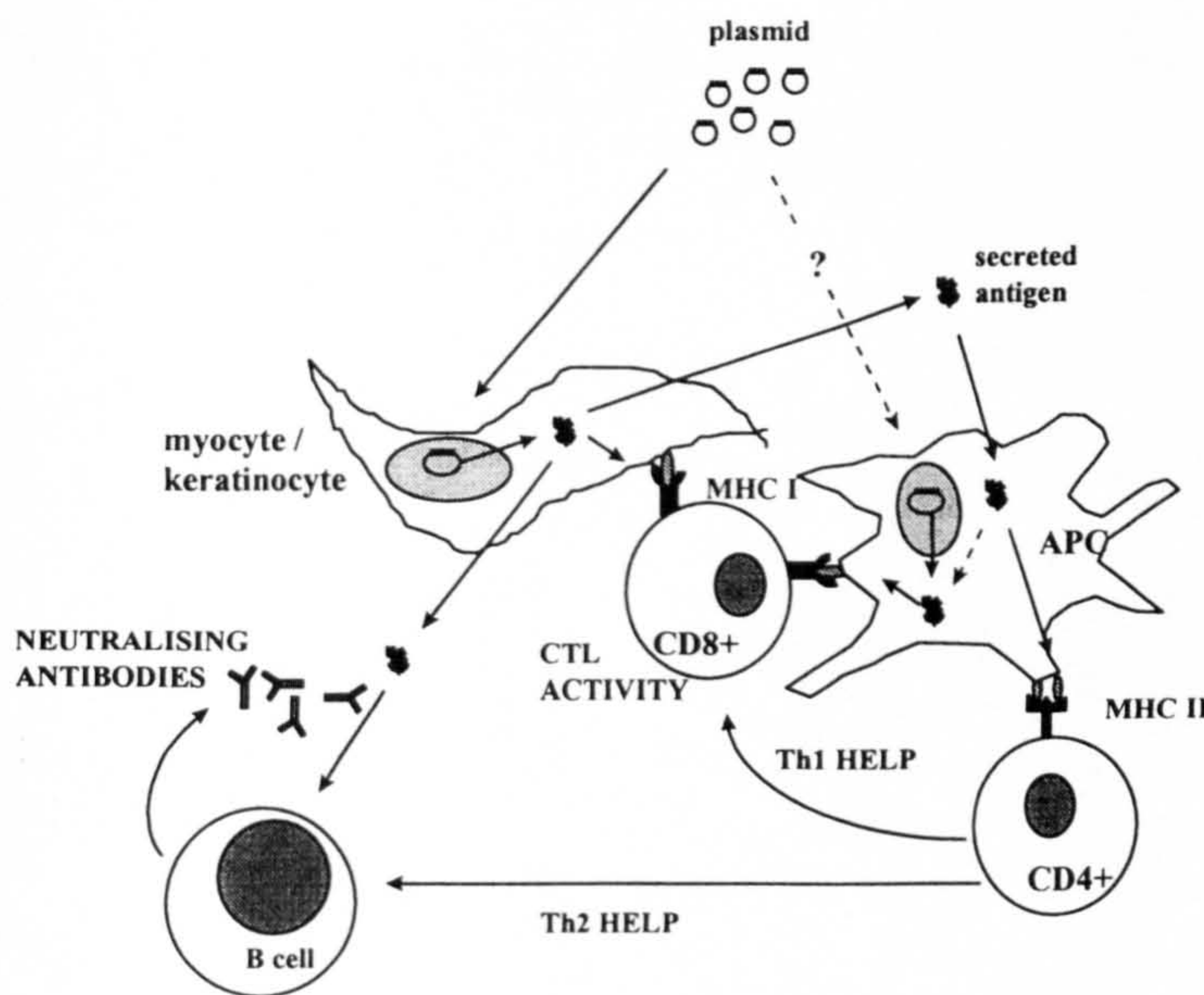


Figure 6.2 Immune responses triggered by DNA immunisation (modified from Cohen *et al*, 1998). The two alternatives for MHC class I presentation by APCs are depicted in broken lines.

In this hypothesis a tissue cell (e.g. myocyte, keratinocyte) is transfected and expresses the encoded protein. The protein is then processed by the cell and presented in the context of MHC class I to CD8+ T cells. However, this assumption is rather dubious since it is now accepted that efficient priming of T cells only occurs after migration of antigen presenting cells to draining lymph nodes. Soluble protein released by the cell can elicit antibody responses and, after phagocytosis and processing by APCs, be presented to CD4+ T cells as MHC class II molecules. At the same time or alternatively, bone marrow derived APCs (e.g. dendritic cells),

infiltrating or resident in the target tissue, can be directly transfected, presenting antigen via MHC class I, while humoral and T helper responses would be generated by soluble antigen produced either by APCs or tissue specific cells (reviewed in Donnelly *et al*², 1997; Robinson and Torres, 1997; Cohen *et al*, 1998). An alternative mechanism in which following internalisation by APCs some peptide antigen might cross-over to the MHC class I pathway is gaining supporters (Kovacsovic-Bankowski and Rock, 1995). This mechanism would imply that transfection of myocytes/ keratinocytes is sufficient to sustain both MHC class I and class II presentation by professional APCs.

6.1.2 Vaccination against influenza virus

Equine influenza vaccines

Current vaccines against equine influenza virus are composed of inactivated whole virus representing strains circulating in the equine population (Mumford *et al*, 1995). One of the major limitations of these vaccines is the failure in maintaining sufficiently high levels of neutralising antibodies between doses, therefore compromising protection (Mumford and Hannant, 1993). Secondly, although major shifts in the antigenic nature of the virus are infrequent in equine influenza, the accumulation of small variations (drifts) that regularly occur in circulating viruses (particularly of the H3N8 subtype) is responsible for the reduced efficacy of the vaccines used. This not only requires a constant antigenic surveillance of the circulating viruses, but, more importantly, dictates the alteration of vaccine formulations every time a significant variation is detected, with all the cost implications that it brings. Recently, the world health organisation (WHO) has made two recommendations to include new variants in equine vaccines. The first, in 1992, for the inclusion of an A/equine2 (H3N8) isolated in 1989 in Britain and the second in 1995 for the inclusion of a Newmarket/2/93-like virus also representing the European group of H3N8 viruses (Mumford and Wood, 1993). Additionally, the unreliability of the method commonly used to measure the antigenic content of vaccine preparations creates difficulties in the correlation of protection with dose of antigen (haemagglutinin) in the vaccine. The method, based

on the ability of HA to agglutinate chick red blood cells, is still used by many vaccine manufacturers, despite recommendations, by the WHO, that a more reproducible assay (single radial diffusion) should be adopted (Mumford *et al*, 1995).

Natural infection by equine influenza virus generates a short lived antibody response that declines to low levels in a period of 3 months. Nevertheless, infection-induced immunity promotes long lasting protection, unlike that induced by vaccination (Hannant *et al*, 1988). Furthermore, following equine influenza infection, both respiratory tract antibody responses in the shape of secretory IgA (sIgA) (Mumford and Hannant, 1993), and CTLs, showing similar MHC class I restriction, to those seen in humans and mice, have been identified in horses (McMichael *et al*, 1983). The evolutionary proximity between equine and human influenza viruses and the similarities in the immune responses elicited imply that the lessons learned from both human vaccinology and that of animal models are useful resources in defining new strategies for equine influenza vaccine development.

Immune response to human influenza vaccines

It has been known for some time that specific immunity to influenza virus is associated with humoral immune responses, both systemic (serum virus neutralising IgG) and of the respiratory tract (virus specific sIgA and IgG antibodies in nasal secretions) and with cell mediated immune responses (reviewed in chapter 5). Antibody responses protect humans against homologous challenge, i.e., with virus possessing the same antigenic specificity (Clements *et al*, 1984) while CTL responses have been associated not only with recovery from infection (with clearance of the virus) but also with protection against heterologous challenge, i.e. following infection with antigenic variants of the virus (reviewed in Ada and Jones, 1986).

Due to the difficulty in securing human individuals naive to influenza virus, most vaccine trials rely on the use of primed recipients and have been conducted in volunteer children, less likely to have had repeated contact with the virus (Ghendon, 1989). In such studies, it has been found that both inactivated vaccines and cold

adapted (temperature sensitive) live attenuated vaccines are extremely efficient in generating neutralising antibodies (Johnson *et al*, 1985). In contrast, stimulation of CTL responses using live vaccines is superior to that obtained with inactivated vaccines (Webster and Askonas, 1980). In the case of inactivated vaccines, responses are highly irregular appearing to be related with the pre-immunisation level of T cell memory. In addition, CTL responses generated by live vaccines were reported to increase protection to heterologous challenge (McMichael *et al*, 1983).

DNA vaccination against influenza in animal models

The limitations imposed by safety considerations and the non availability of unprimed human individuals have prompted the pursuit of animal models that would allow for immune responses to be correlated with protective efficacy of human influenza vaccines in preclinical studies. Some animal models (e.g. ferrets) are readily infected with strains of human influenza, however, due to practicability and cost the murine model continues to be preferred. Because most human influenza strains are not able to naturally infect mice, the generation of murine challenge models had to rely on the adaptation of virus to this species. Such is the efficiency of these models to respond to influenza virus, that high morbidity and even death can be induced with appropriate doses of challenge virus (reviewed in Donnelly³ *et al*, 1997). Infection of mice with adapted virus in sublethal doses leads to the development of protective antibody and cell mediated responses. These models have been particularly useful in the dissection of the involvement of CTLs in recovery from infection and protection to influenza challenge, by allowing experiments such as the treatment with anti-CD8⁺ antibodies, the knockout of the β 2-microglobulin gene or the adoptive transfer of CTL clones from infected mice to severely compromised immunodeficient (SCID) mice, that lack mature T and B cells. Similar experiments were performed in relation to Th1 clones that were also found to be important for protection against influenza infection (reviewed in Donnelly³ *et al*, 1997).

An initial report, on DNA vaccination against human influenza virus in mice, showed that intramuscular injection of plasmid encoding nucleoprotein (NP) generated NP

specific CTLs and protection against lethal heterologous challenge (Ulmer *et al*, 1993). These results were confirmed and expanded to demonstrate that plasmid encoding haemagglutinin (HA) promoted high titres of anti-HA antibodies that protected mice from lethal homologous challenge (Montgomery *et al*, 1993). Subsequently Donnelly *et al* (1995) have carried out similar experiments in ferrets with comparable results. Because ferrets can be naturally infected by human influenza, they exhibit an analogous pattern of pathogenesis that includes viral shedding. In this study HA DNA vaccinated ferrets presented significantly reduced shedding following homologous challenge, when compared to control animals. In the same study, the authors used non-human primates to compare the efficacy of DNA vaccines to that of current vaccines licensed for human use. They found that the injection of as little as 10µg of HA encoding plasmid induced titres of haemagglutination inhibition equal or higher than those produced by a full dose of human vaccine.

In summary, DNA vaccination with influenza HA and NP genes generates high titres of virus neutralising antibodies (as assessed by haemagglutination inhibition) that protect from homologous challenge (with reduction of clinical signs and virus shedding) as well as specific cell mediated responses (capable of lysing target cells infected with influenza virus) protective against heterologous challenge in both mice and ferrets, constituting a viable alternative for current human vaccines, as determined for non-human primates. If these results can be verified in natural hosts of influenza (e.g. human, horse) they will dictate a revolution in the vaccination against these viruses, for two main reasons. Firstly the efficient homologous protection conferred by vaccination with the HA gene, together with the ease of production and standardisation of these vaccines, means that influenza vaccines can be quickly and inexpensively updated. Secondly, the cross-strain protection obtained by vaccination with the NP gene will reduce the need for constant vaccine update.

6.1.3 DNA vaccination of horses against influenza virus

In the light of findings, chiefly in mice but also in other species, that plasmid DNA innoculation of antigens is an efficient way of protecting hosts from influenza virus challenge, its reasonable to assume that this technology will also be applicable to horses. Results by Donnelly *et al* (1995) and others, suggest that the HA and NP antigens are useful in the immunisation against influenza virus and these were chosen to serve as immunogens in an equine influenza model of DNA vaccination. In addition, the cloning of a variety of equine cytokines, particularly those involved in Th1 responses (chapters 3 and 4), has facilitated their inclusion in the vaccine trial, ensuing reports that these molecules, when co-administrated with plasmid encoding antigen, can significantly increase cell mediated immunity without affecting humoral responses to soluble antigens (reviewed in chapter 1).

Below is a description of a feasibility study for the DNA vaccination of horses against influenza virus, where the efficacy of these vaccines is compared to that of a classical vaccine, composed of whole inactivated virus strains. The optimisation of factors such as route of inoculation, co-administration of different combinations of cytokines and use of adjuvant vehicles is pondered.

6.2 Materials and Methods

The present vaccine trial was a collaborative study between the Department of Veterinary Pathology (Glasgow, UK), Intervet Virology R&D (Boxmeer, Netherlands) and Intervet Magyar (Budapest, Hungary). The responsibility of the Department of Veterinary Pathology in this study was the production of the plasmid DNA constructs used for inoculation and evaluation of the authenticity of the genes inserted. This fact is reflected in the description of the methods. An account of the methods performed elsewhere was distributed in memorandums by Intervet International and is summarised below, and the names of those involved acknowledged, when known.

6.2.1 DNA constructs

The DNA constructs used for vaccination purposes were based on the pCI-neo expression vector (Promega) by insertion of the coding sequences of the equine cytokines IFN- γ (as described in chapter 3), IL-12 and IL-18 (chapter 4) and of the influenza antigens HA and NP derived from the isolate A/equine/Newmarket/2/93 of the H3N8 subtype (chapter 5). The pCI-neo plasmid features include a cytomegalovirus immediate early (CMV-IE) enhancer/promoter with a chimeric intron that drives expression of the inserted genes, a SV40 late polyadenylation signal sequence for mRNA processing, a fl origin of replication and an ampicillin resistance gene for replication and selection in *E.coli*.

After production, the constructs were sequenced for verification of integrity of the genes inserted and tested for the ability to express the encoded proteins by transient transfection of appropriate cell lines. Investigation of biological activity (where applicable) of the expressed products was also attempted. At the time of the vaccine trial, the status of the DNA constructs in these regards is presented in table 6.1.

Table 6.1 Verification procedures of DNA constructs.

Construct	Procedure			
	Sequencing	Detection of expression		Bioassay
		RNA	Protein	
pCI-neo	n.a.	n.a.	n.a.	n.a.
pCI-IFN- γ	+	+	+	+
pCI-IL12	+	+	n.d.	n.d.
pCI-IL18	+	+	+	n.d.
pCI-HA	+	+	+	n.a.
pCI-NP	+	+	+	n.a.

Notes: n.a.- not applicable; n.d.- not done. Detection of RNA expression was done by northern blot. Detection of protein expression was done by western blot in the case of IFN- γ and IL-18 and by indirect immunofluorescence of transfected cells for HA and NP (Intervet, Boxmeer). For details on results refer to individual chapters.

6.2.2 Trial design

In the experimental design, conditions such as the vaccination schedule, inoculation dose, and route were based on parameters used for similar studies in other animal species (reviewed in 6.1). Thirty horses (see 6.2.4) were randomised and assigned to eight vaccination groups of 3 to 4 horses each. Assignment of horses to groups and respective vaccines are shown in table 6.2 and a diagram of the trial is displayed in figure 6.3.

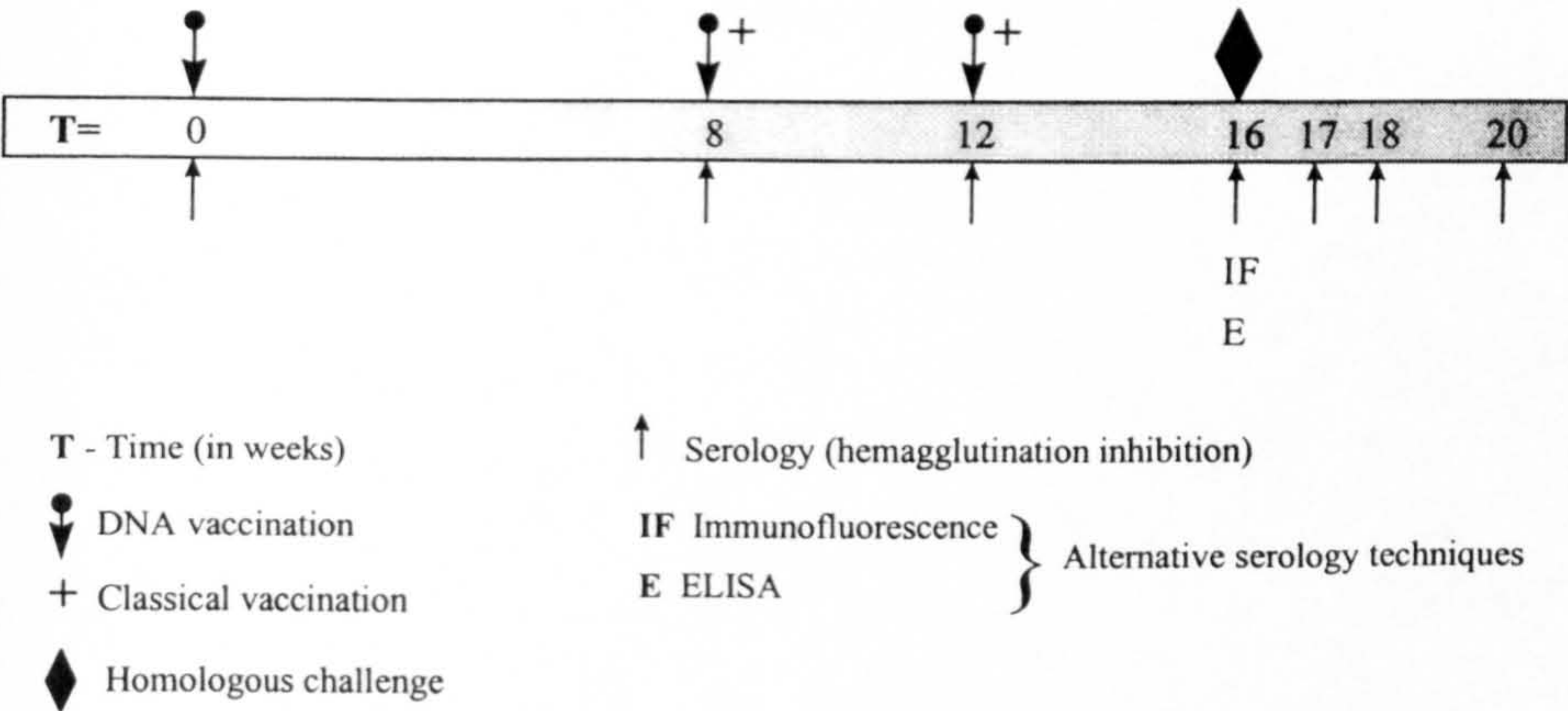


Figure 6.3 Schematic of vaccine trial showing vaccination, challenge and sampling timings.

Six groups received DNA vaccinations consisting of plasmid encoding HA and NP, co-administered or not with plasmids encoding different cytokines. Animals were

vaccinated three times at $t = 0, 8$ and 12 weeks. A group of 4 animals was inoculated, at the same times, with plasmid lacking a coding region (empty plasmid) to serve as negative control. A further group of 4 animals received a classical subunit vaccine at times $t = 8$ and 12 weeks. The vaccines (refer to 6.2.3 for formulations) were inoculated in 1ml volumes by one of the following routes (table 6.2), intradermally (i.d.) at five different spots ($5 \times 200\mu\text{l}$), by means of a needleless intradermal injector (IDAL, Intervet) or intramuscularly (i.m.) by injection using a 2ml syringe with green needle (21 gauge).

Table 6.2 Experimental groups and respective vaccine tests.

Group no	No. of horses (assigned no.)	Vaccine	Vehicle	Route (a)	Dose (b)
1	4 (18,21,26,30)	HA+NP	PBS	i.d.	$2 \times 100\mu\text{g}$
2	3 (2,22,28)	HA+NP	Diluvac Forte	i.d.	$2 \times 100\mu\text{g}$
3	4 (12,15,23,24)	HA+NP+IFN γ	PBS	i.d.	$3 \times 100\mu\text{g}$
4	3 (9,11,16)	HA+NP+IFN γ	PBS	i.m.	$3 \times 100\mu\text{g}$
5	4 (5,14,27,29)	HA+NP+IL18	PBS	i.d.	$3 \times 100\mu\text{g}$
6	4 (8,10,13,20)	HA+NP+IL12+IL18	PBS	i.d.	$4 \times 100\mu\text{g}$
7	4 (1,4,17,19)	empty plasmid	PBS	i.d.	$500\mu\text{g}$
8	4 (3,6,7,25)	classical vaccine	Buffer + QuilA	i.m.	-

(a) intradermal (i.d.) or intramuscular (i.m.). (b) in a total volume of 1ml per dose.

All animals were challenged, at $t = 16$ weeks, with a homologous influenza virus strain (A/equine Newmarket/2/93). The contents of one vial of freeze-dried virus (refer to 6.2.3) was resuspended in 5ml of ice cold PBS and transferred to a disposable chamber in the ultrasonic nebuliser (Ultra-Neb 2000, Devilbis). The virus was then applied to the horses by nebulisation using a head hood.

6.2.3 Inoculates

DNA VACCINES

Following characterisation, all constructs were propagated in the *E. coli* DH5 α strain and prepared by plasmid DNA large scale purification procedures and in all cases endotoxin levels were found to be inferior to 20 EU/100 μg of DNA (performed by Dr.

P. Sondermeijer, Intervet, Boxmeer). The vaccine preparations were re-sequenced to rule out the occurrence of mutations during the replication process. Different vaccine preparations (table 6.2) were produced by combination of appropriate plasmid DNAs (100µg each) and dilution to a total volume of 1ml, per dose, in endotoxin-free PBS or Diluvac Forte and stored at -20°C. Diluvac Forte is an adjuvant formulation based on tocopherol acetate patented by Intervet (see below).

CLASSICAL VACCINE

The vaccine (produced by Dr. H. Powels *et al*, at Intervet, Boxmeer) comprised inactivated viral subunits adjuvanted by Quil A. The viral strains A/equine/Prague/56, Newmarket/1/93 and Newmarket/2/93, with a HA content of 928, 1000 and 1000 antigenic units, respectively (as assessed by standard antigen ELISA) were added to 125µg of Quil A and diluted to a total volume of 1 ml, per dose, in Nobi-Equenza vaccine buffer (Intervet patented formulation) and stored at 4°C.

CHALLENGE MATERIAL

The challenge virus (A/equine/Newmarket/2/93) was propagated by repeated passage in specific pathogen free (SPF) embryonated chicken eggs to an infectivity titre of 10^6 to $10^{6.5}$ EID₅₀/ml (as assessed by haemagglutination of chicken red blood cells, CRBC). Vials containing 1ml of virus suspension were freeze-dried and stored until used at -20°C (Dr. E. Verblakt *et al*, Intervet, Boxmeer).

6.2.4 Animal husbandry and veterinary control

Thirty warm-blooded, Hungarian half-bred horses (27 males and 3 females) with ages ranging 9-13 months, were purchased. Before purchase, the horses were found to be seronegative to influenza virus in two to three successive screenings. On arrival the animals were numbered (1 to 30) and identified (by numbered tags), randomised (using “Random Generator” computer software), assigned to vaccination groups and housed in a designated stable (at the Budapest School of Veterinary Medicine, Hungary). The diet consisted of meadow hay *ad libitum*, oat (2-3 kg daily), carrots

(0.5-1 kg daily, if available) and tap water *ad libitum*. Before the trial the animals were acclimatised for a period of two weeks.

The horses were subject to a daily clinical examination to assess general state of health. From two days before and until five days after vaccine applications, horses were submitted to a thorough veterinary inspection including detection of body temperature, inappetance, loss of appetite, abnormal behaviour, sickness, malaise and other abnormal signs. From the day of vaccination and for five days, local reactions were scored by size (cms) and by palpation (cold/warm, painful/not painful, hard/soft). Also from two days before and up to seven days after challenge animals were examined for evidence of abnormal behaviour, inappetance, sickness, malaise, coughing, nasal discharge and dyspnoea. Animal handling and veterinary examinations were performed by Dr. J. Foldi *et al* (Intervet Magyar).

6.2.5 Sampling and analyses

SAMPLING

Blood samples for serology were taken at $t = 0, 4, 8, 12, 16, 17, 18$ and 20 weeks. Blood (10 ml) was collected from the jugular vein into gel-filled, vacuum-sealed, plastic tubes and the tubes were labelled with the date and respective animal number. The blood was left to clot for four hours at room temperature (or overnight at 4°C), the tubes centrifuged for 10 min. at 1500 x g to pellet cells and despatched to Intervet (Boxmeer) as soon as possible at 4°C. Alternatively, when more convenient, following centrifugation, serum was transferred to polypropylene tubes (cryotubes), which were labelled and stored at -20°C until transport.

Swabs for virus isolation were collected from day 1 up to day 7 post-challenge. A separate swab was used for each nostril and transferred to cryotubes containing 1.5 ml of 2.5% tryptose and antibiotics (1000 IU/ml of penicillin and 1000 µg/ml of streptomycin). Tubes were stored at -70°C before transport to Boxmeer.

ANALYSES

All analysis were performed by Dr. H. Pouwels *et al*, at Intervet virology R&D (Boxmeer) and the protocols presented summarised from Intervet's standard operating procedures.

Serology was mainly performed by titration of serum by haemagglutination inhibition against four HA antigenic units of the strain A/equine/Newmarket/2/93. In summary, serum samples are pre-treated in kaolin, mixed with a 5% CRBC suspension and incubated at 37°C for 1 hour. Tween/ether treated equine influenza antigens are diluted to 4 haemagglutinin units and added to the first row of a microtitre plate (v-shaped wells). The haemagglutinin is diluted in twofold increments and the pre-treated serum-CRBC mixture added to the plate. Following 1 hour incubation at room temperature the plates are scored by naked eye inspection for the presence of agglutination in individual wells. The assay is performed in triplicate and the quantity of haemagglutination inhibiting (HI) antibodies, named HI titre, is expressed as $-\log_2$ of the sample dilution. For samples collected at $t = 16$ weeks, two additional serological procedures were employed. The first was a blocking-antibody-ELISA, using a mAb raised against the haemagglutination inducing site of the HA protein of strain A/equine/Newmarket/2/93 and the second, an indirect immunofluorescence assay on Madin Darby bovine kidney (MDCK) cells infected with A/equine/Newmarket/2/93.

Virus in swabs was detected and titrated in 9-11 day old embryonated SPF chicken eggs. In short, five eggs were used per each of 5 tenfold dilutions of the swabs. Eggs were inoculated, in the allantoic cavity, with 200 μ l of corresponding swab dilution and incubated at 37°C in a humidified egg incubator for 72 hours. Embryo death in the first 24 hours was considered unspecific and the eggs discarded. After incubation, allantoic fluid was harvested from each egg and added to an equal amount of 5% CRBC for detection of haemagglutination and calculation of virus titre. The titre is given in egg infectious dose ($\text{EID}_{50}/\text{ml}$) using the formula:

$$\text{Titre} = 10^A \times 10^{(B-50)/(B-C)} \text{EID}_{50}/0.2\text{ml},$$

where A = first dilution at which more than 50% of the eggs is positive; B = percentage of positive eggs above 50%; C = percentage of positive eggs below 50%.

6.3 Results

CLINICAL SIGNS

Veterinary observation of the animals during the period of trial did not detect any abnormalities in clinical signs that could be correlated with both the vaccinations and the challenge.

SEROLOGY

Serological responses were monitored by haemagglutination inhibition (HI) of the trial horses and the results are given in antibody log₂ titre. Treatment of the data revealed that, in general, the DNA vaccinated groups had a poor response to inoculation, in terms of neutralising antibody production. This was confirmed both by the higher antibody levels, pre-challenge, presented by the group vaccinated with inactivated virus (group 8) and by the fact that, following challenge, all groups had a significant rise in the average titres of antibodies (table 6.3, figures 6.4 and 6.5) presumably triggered by the challenge virus. It was also found that the administration of vaccine by an alternative route (i.m., group 4) or the use of a classical adjuvant (Diluvac Forte, group 2) did not affect the antibody production when compared with groups 3 and 1, respectively.

However, analysis of the results in individual animals revealed that trial horses can be divided into three groups: responsive to vaccination, non-responders and responders to challenge, the basis of the division being the time (in weeks) at which a clear elevation in antibody titres is observed (table 6.3) and assuming that all trial horses were equally exposed to the challenge virus. Horses responsive to vaccination (with an elevation of titre before challenge) are all animals vaccinated with classical vaccine (group 8) and numbers 18, 23 and 16 respectively from groups 1, 3 and 4. Non-responsive horses (with no significant elevation of antibody titres and specified by an arrow in table 6.3) are present in all groups except group 8. Late responders (with titres raising only after challenge) are also evenly distributed between the groups including the one that served as negative control (group 7).

Also, horses numbers 18, 23 and 16, respectively from groups 1, 3 and 4, had HI-titres pre-challenge that not only were higher than the average of the respective group but were also in line with average titre of the group vaccinated with classical vaccine. Additionally, the antibody titre of animals 18 and 23 was not increased following challenge (figure 6.6).

Results obtained by alternative serological procedures on week 16 (table 6.4) revealed that group 3, had on average antibody titres (as assessed by ELISA) superior to the rest of the groups, although still inferior to the values observed in animals receiving inactivated vaccine. Individual analysis of the ELISA results were even more encouraging, showing that horses 18 (of group 1), 23 (of group 3), 16 (of group 4) 5 (of group 5) and 13 (of group 6) had antibody titres in line with those of the group vaccinated with classical vaccine, whereas animals in the control group had negligible antibody titres. Indirect immunofluorescence showed that all horses of group 3, as well as the above cited animals in the other groups had positive serum as did the ones in the positive control group (group 8). Once again all sera from the negative control group were negative to immunofluorescence.

VIRUS ISOLATION

Virus isolation from swabs collected post-challenge was largely negative (results not shown). Most importantly, in the unvaccinated group only 2 of 4 animals were virus isolation positive with the maximum virus titre obtained being $10^{3.2}$ EID/ml, for horse 17, at day 3 post-challenge.

Table 6.3 Haemagglutination inhibition results. The time of challenge (T = 16 weeks) is highlighted.

Group	Vaccine	Animal	Log ₂ titre against A/equine/Newmarket/2/93							
			T0	T4	T8	T12	T16	T17	T18	T20
1	HA+NP	➡ 30	3	3	3	3	4	3	3	3
		18	3	3	3	3	5	5	5	4
		➡ 21	3	3	3	3	3	4	4	4
		26	3	3	3	3	3	5	7	7
		average	3.0	3.0	3.0	3.0	3.8	4.3	4.8	4.5
2	HA+NP (Diluvac Forte)	2	3	3	4	3	3	3	9	8
		22	3	3	3	3	3	3	3	9
		28	3	3	3	3	3	3	3	8
		average	3.0	3.0	3.3	3.0	3.0	3.0	5.0	8.3
3	HA+NP+IFN _γ	12	3	3	3	3	4	4	5	8
		15	3	3	3	3	3	5	7	8
		23	3	3	3	5	7	7	6	6
		24	3	3	3	3	3	4	4	5
		average	3.0	3.0	3.0	3.5	4.3	5.0	5.5	6.0
4	HA+NP+IFN _γ (Intramuscular)	9	3	3	3	3	3	4	8	7
		11	3	3	3	3	3	3	4	d
		16	3	3	3	3	6	6	9	9
		average	3.0	3.0	3.0	3.0	4.0	4.3	7.0	8.0
5	HA+NP+IL18	5	3	3	3	4	4	6	9	9
		14	3	3	3	3	3	8	11	9
		➡ 27	3	3	3	3	3	3	3	3
		➡ 29	3	3	3	3	3	3	3	5
		average	3.0	3.0	3.0	3.3	3.3	5.0	6.5	6.5
6	HA+NP+IL12+IL18	8	3	3	3	3	3	6	10	9
		10	3	3	3	3	3	4	3	7
		13	3	3	3	3	3	7	11	8
		➡ 20	3	3	3	3	3	3	3	4
		average	3.0	3.0	3.0	3.0	3.0	5.0	6.8	7.0
7	empty vector	4	3	3	3	3	3	3	5	5
		17	3	3	3	3	3	4	8	8
		➡ 19	3	3	3	3	3	3	3	3
		1	3	3	3	3	3	3	9	8
		average	3.0	3.0	3.0	3.0	3.0	3.3	6.3	6.0
8	classical vaccine	3	3	3	3	3	7	8	8	7
		6	4	4	4	4	6	8	7	7
		7	3	3	3	3	7	9	9	8
		25	3	3	3	3	7	7	9	10
		average	3.3	3.3	3.3	3.3	6.8	8.0	8.3	8.0

TX - time = X weeks; d - animal died. ➡ - non responders.

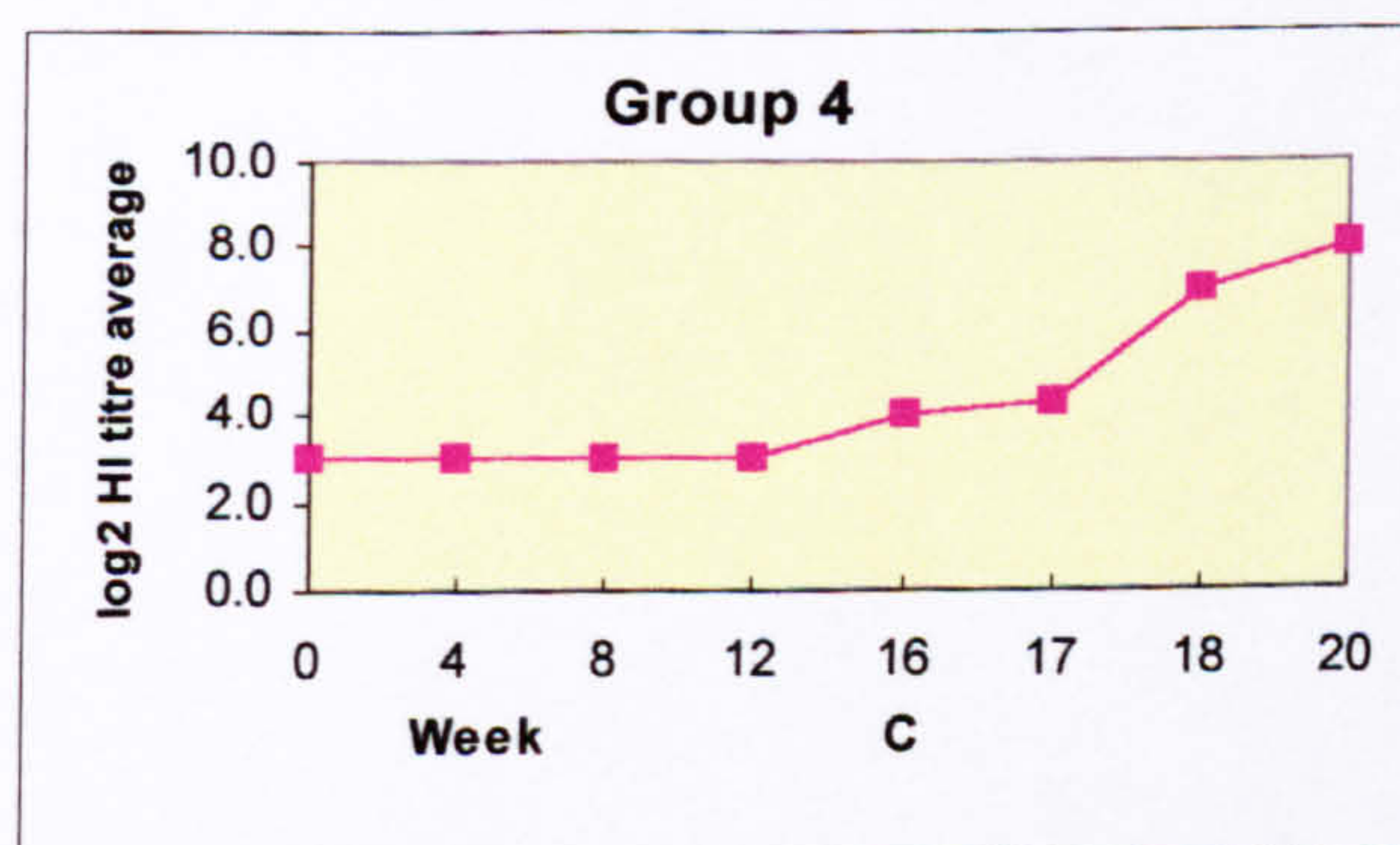
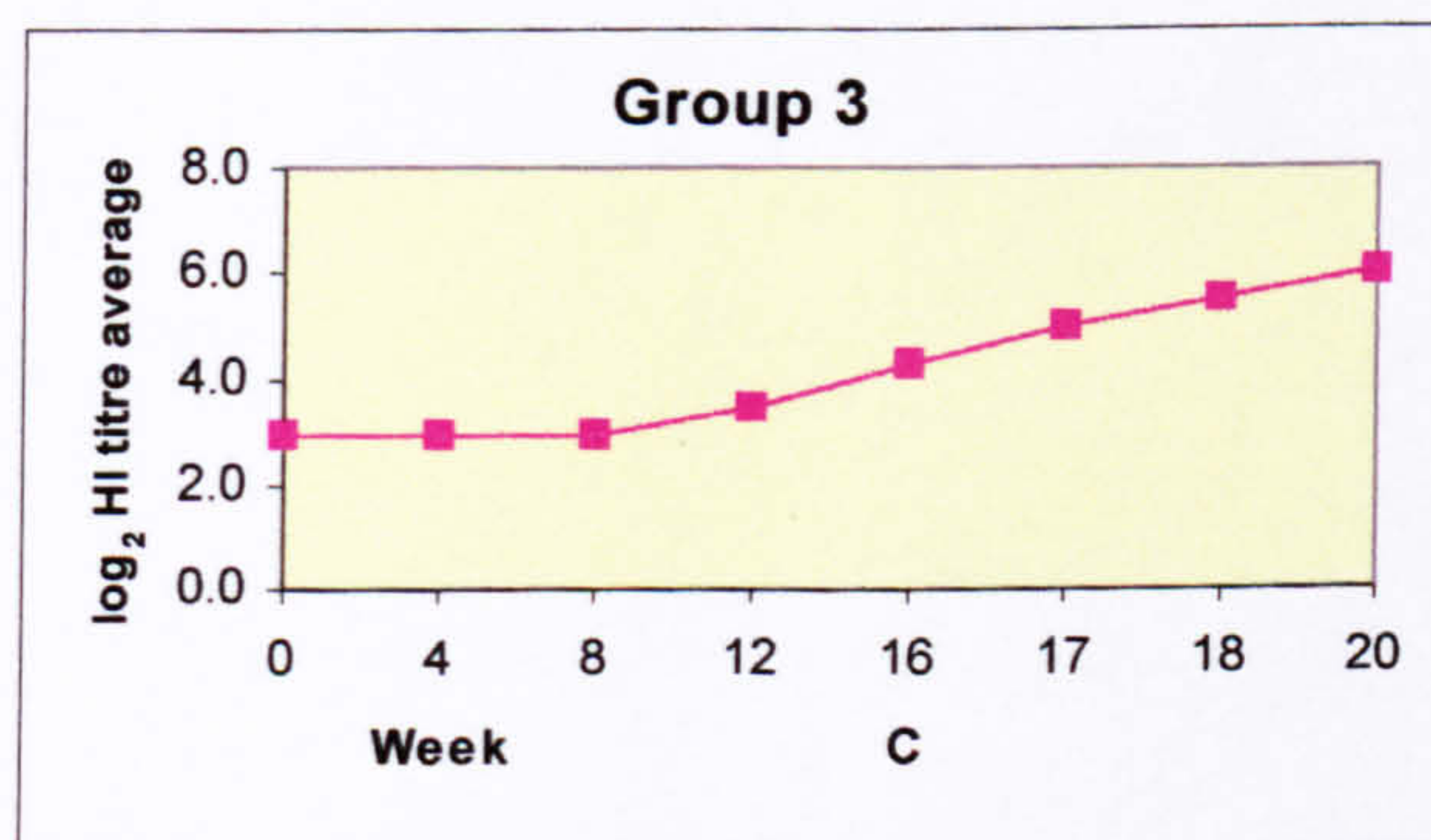
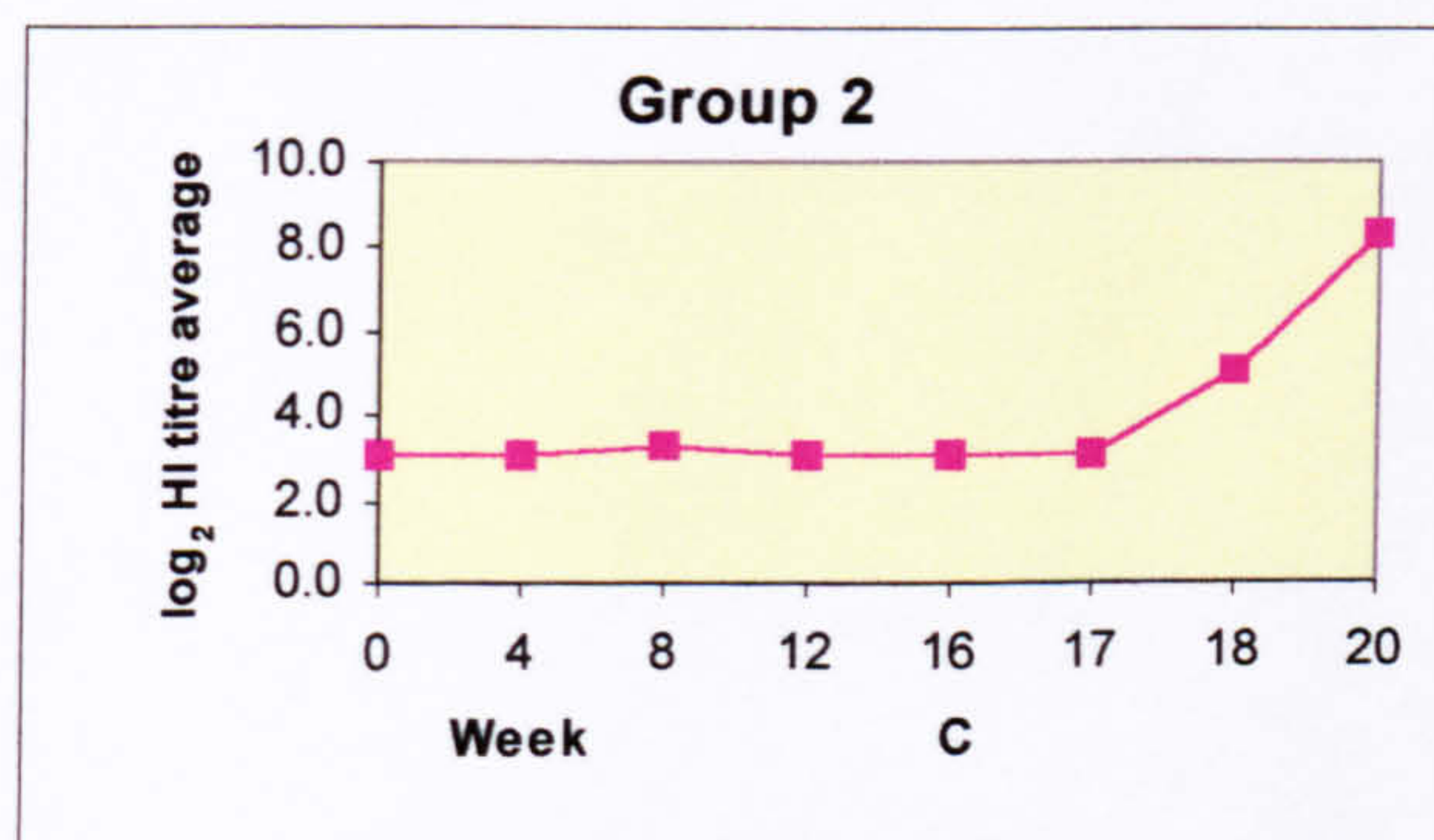
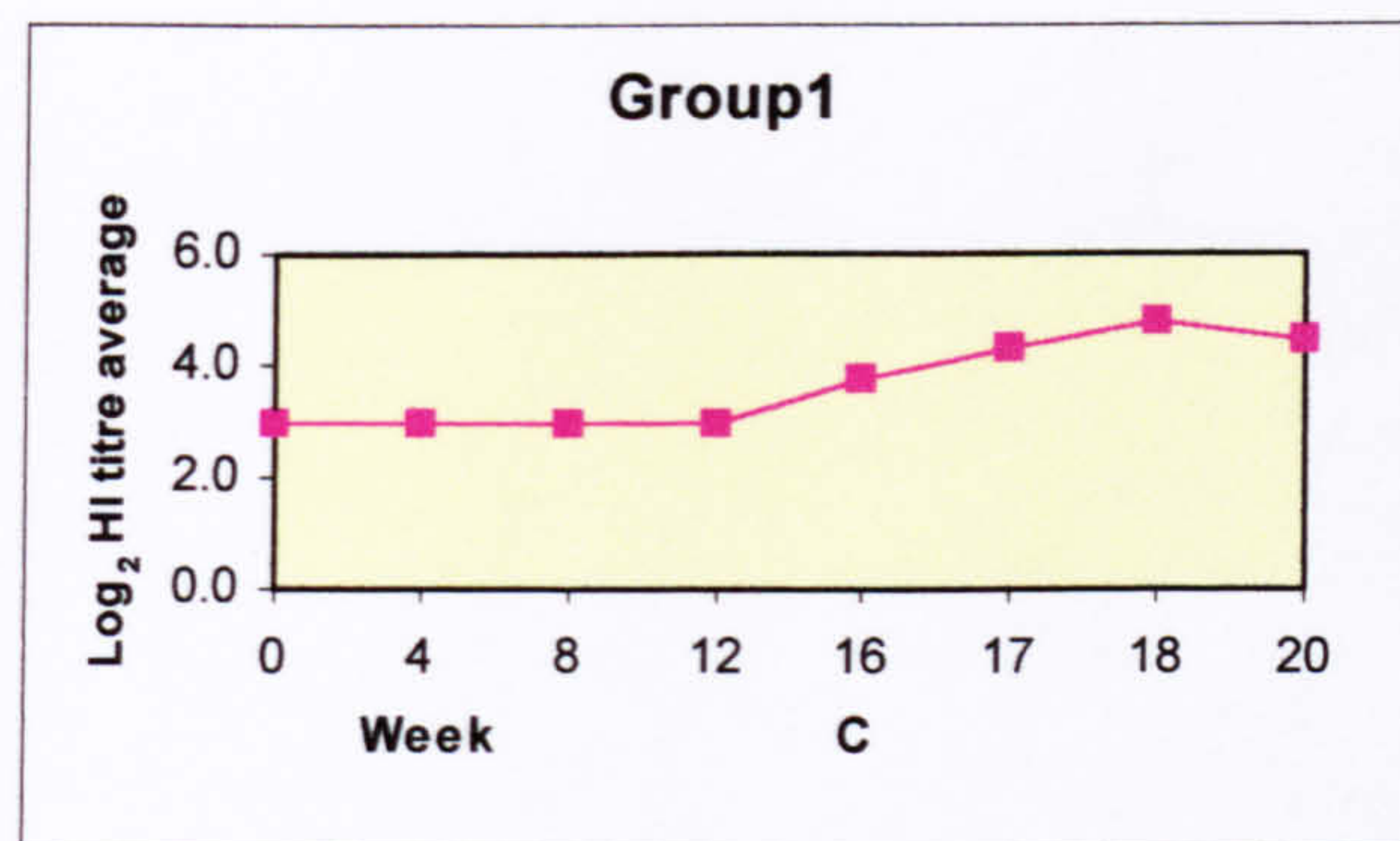


Figure 6.4 Average titres of haemagglutination inhibition antibodies per group. C - time of challenge. (continued overleaf).

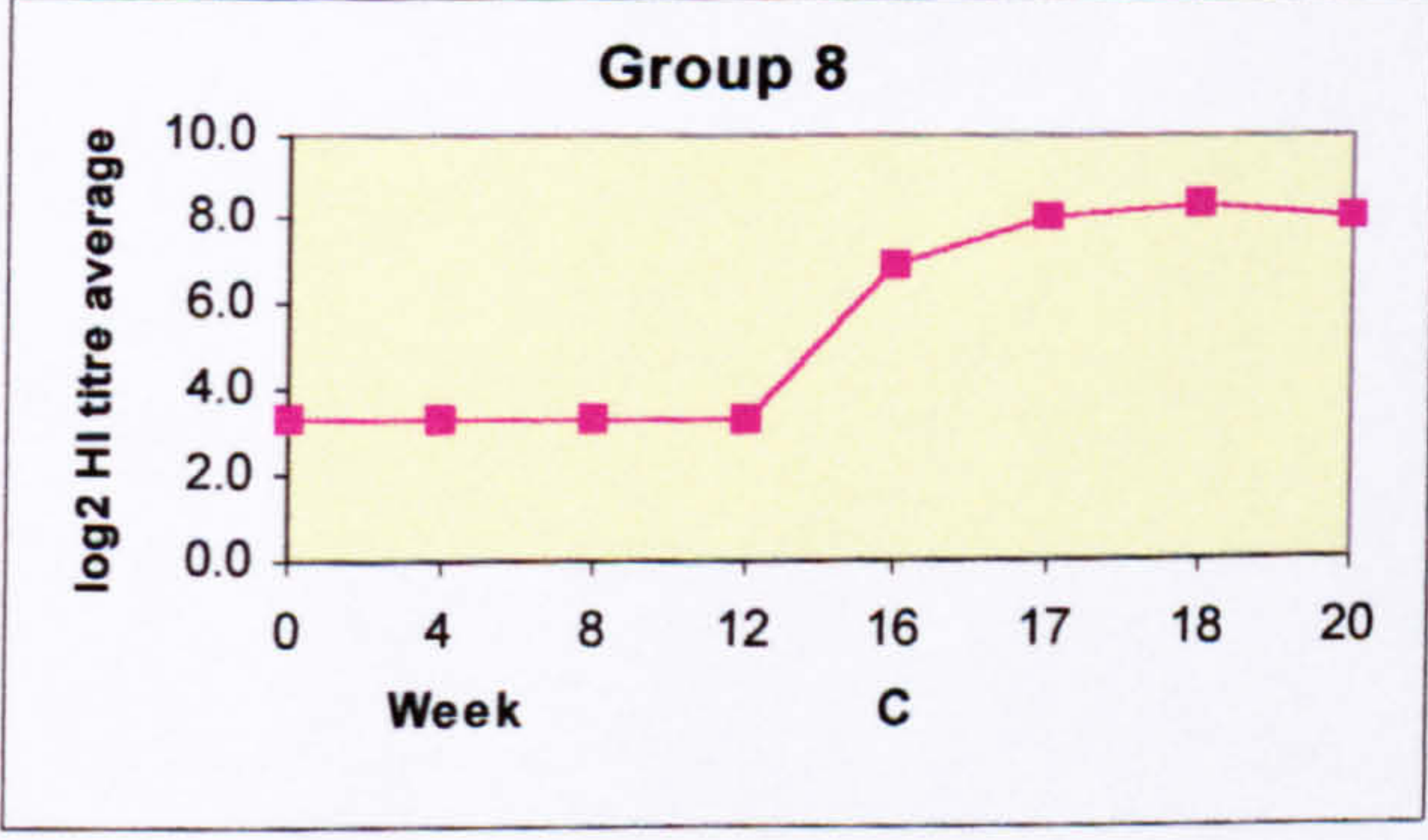
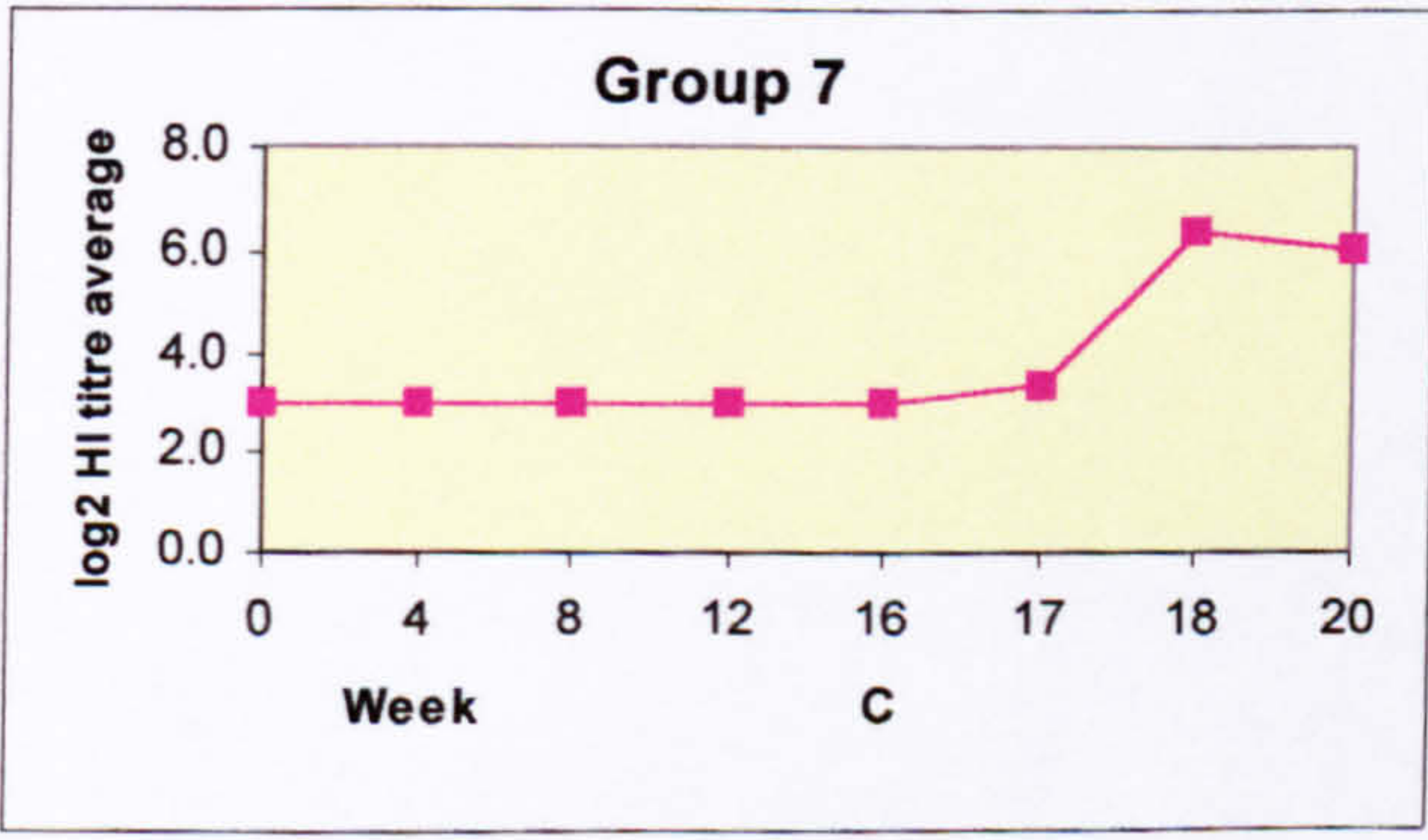
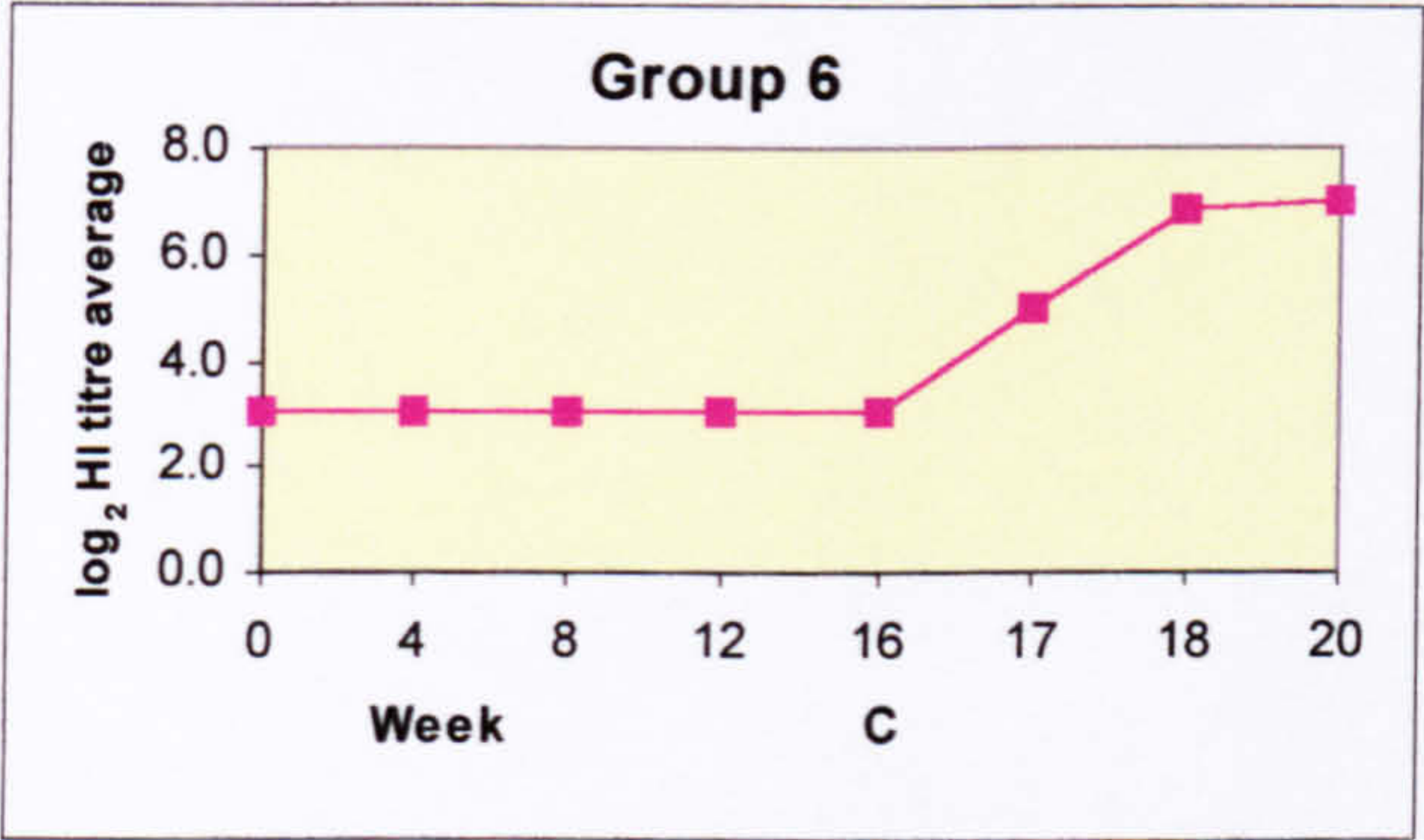
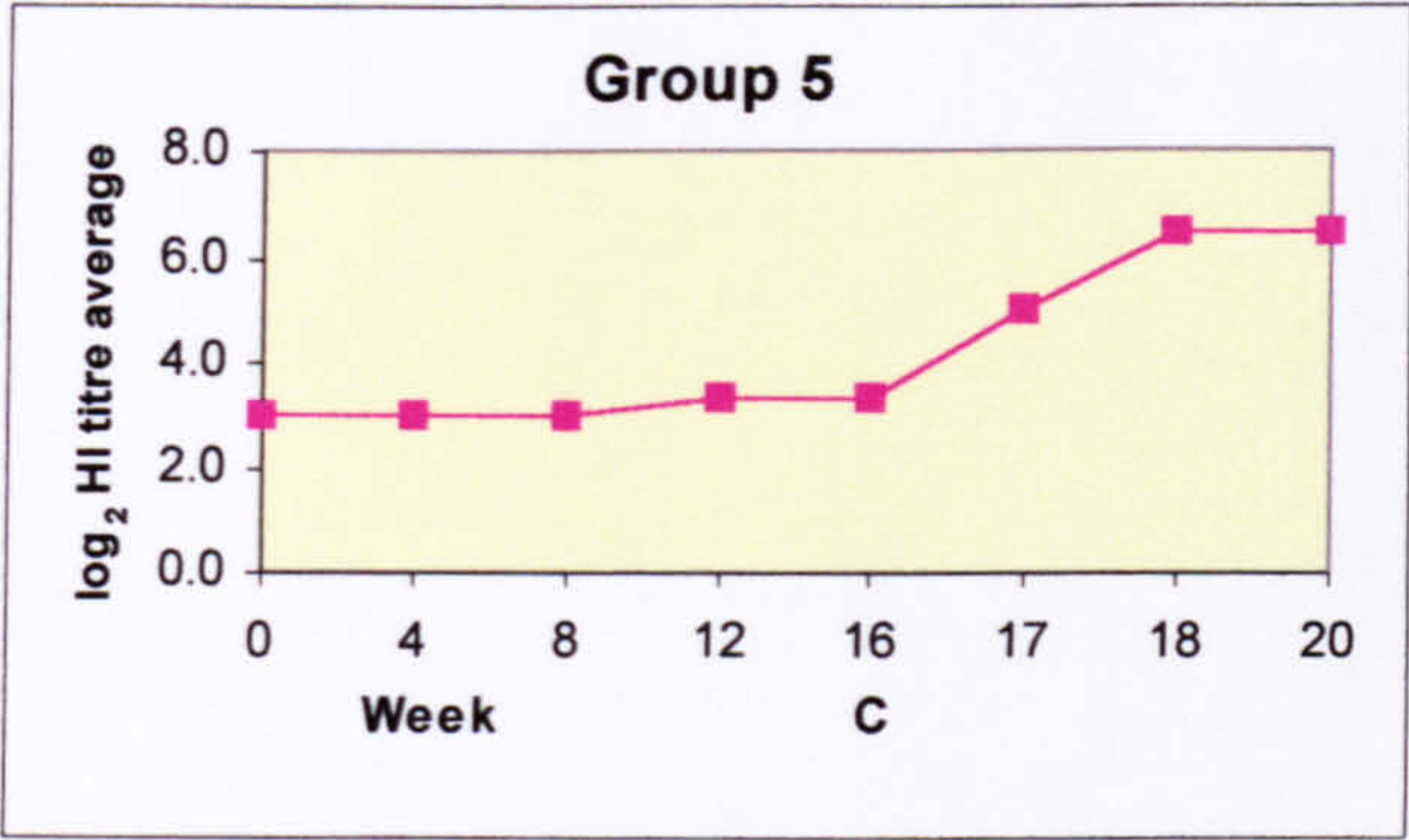


Figure 6.4 (continued from previous page).

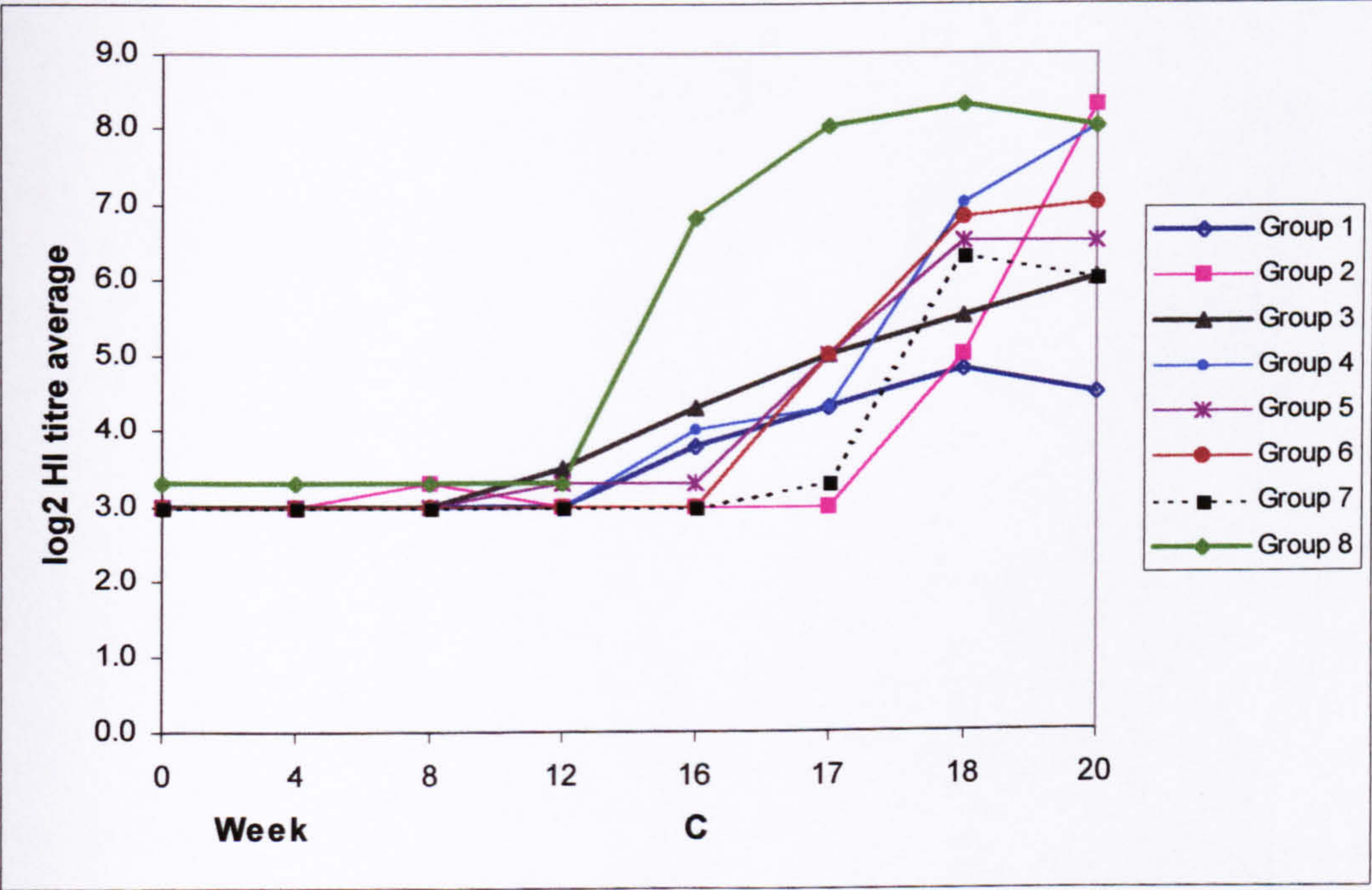


Figure 6.5 Plot of the average HI titre of all vaccination groups. C- time of challenge

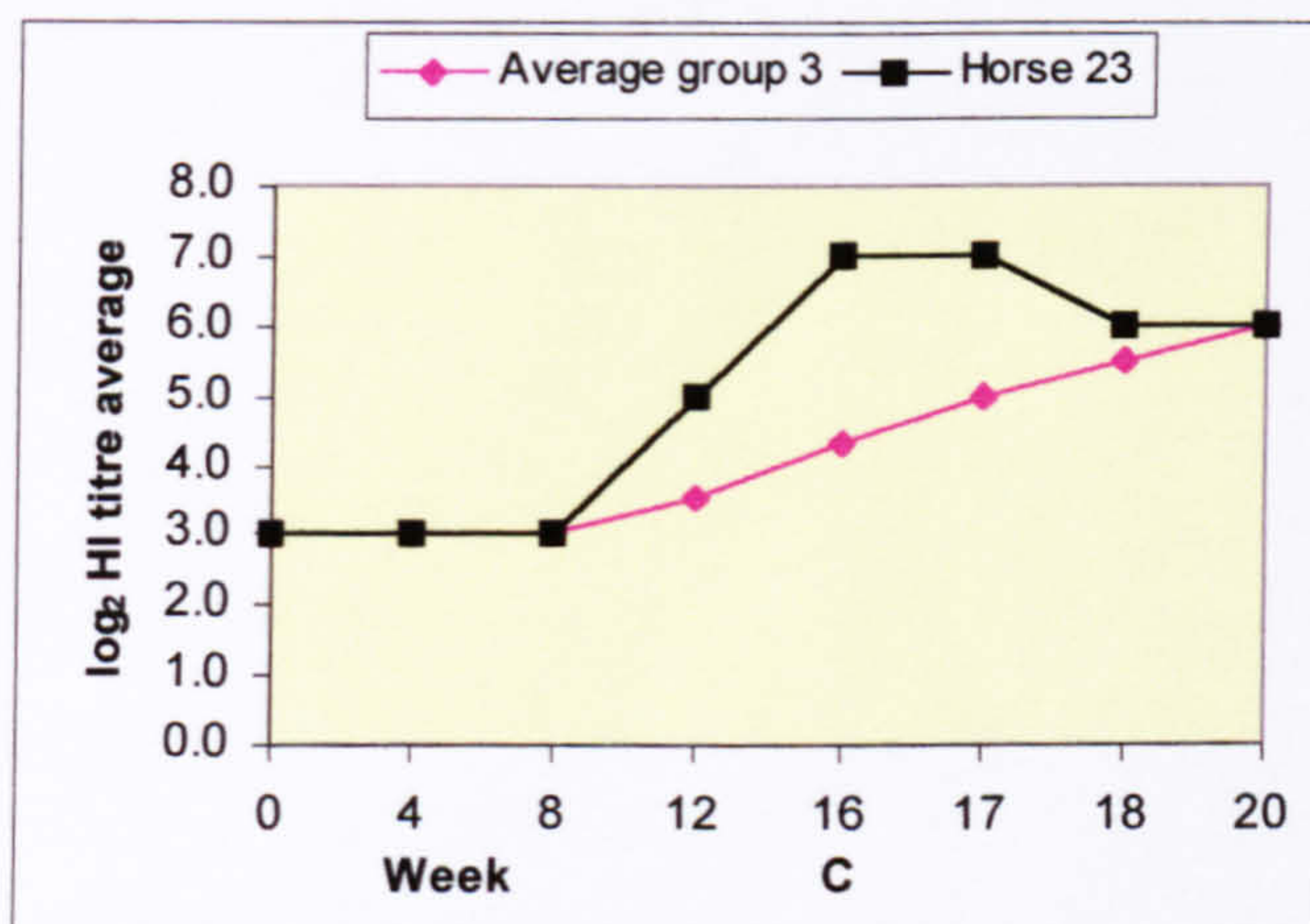
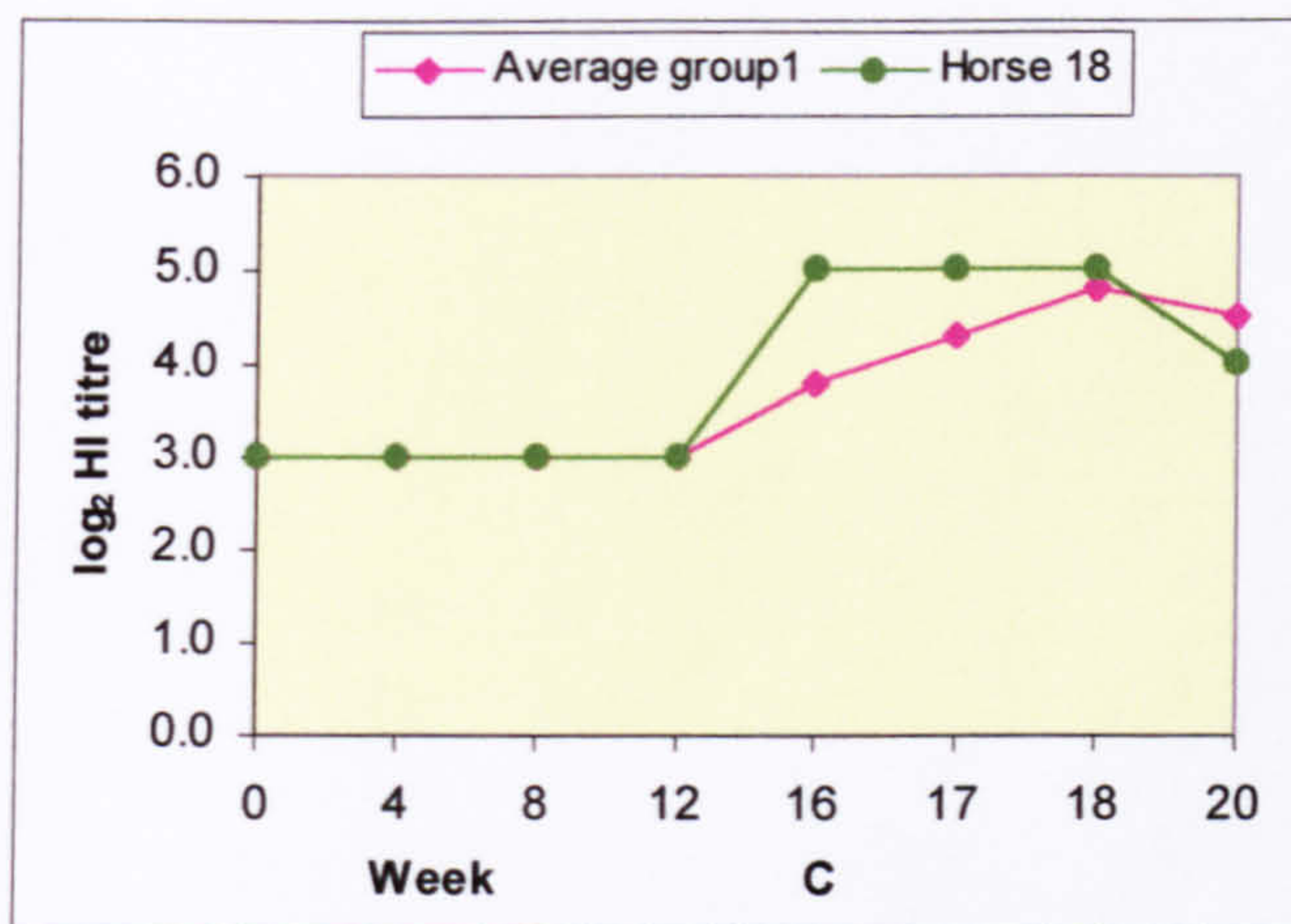


Figure 6.6 Comparison of HI titres of horses 18 and 23 with the group average (group 1 and group 3, respectively). C- time of challenge.

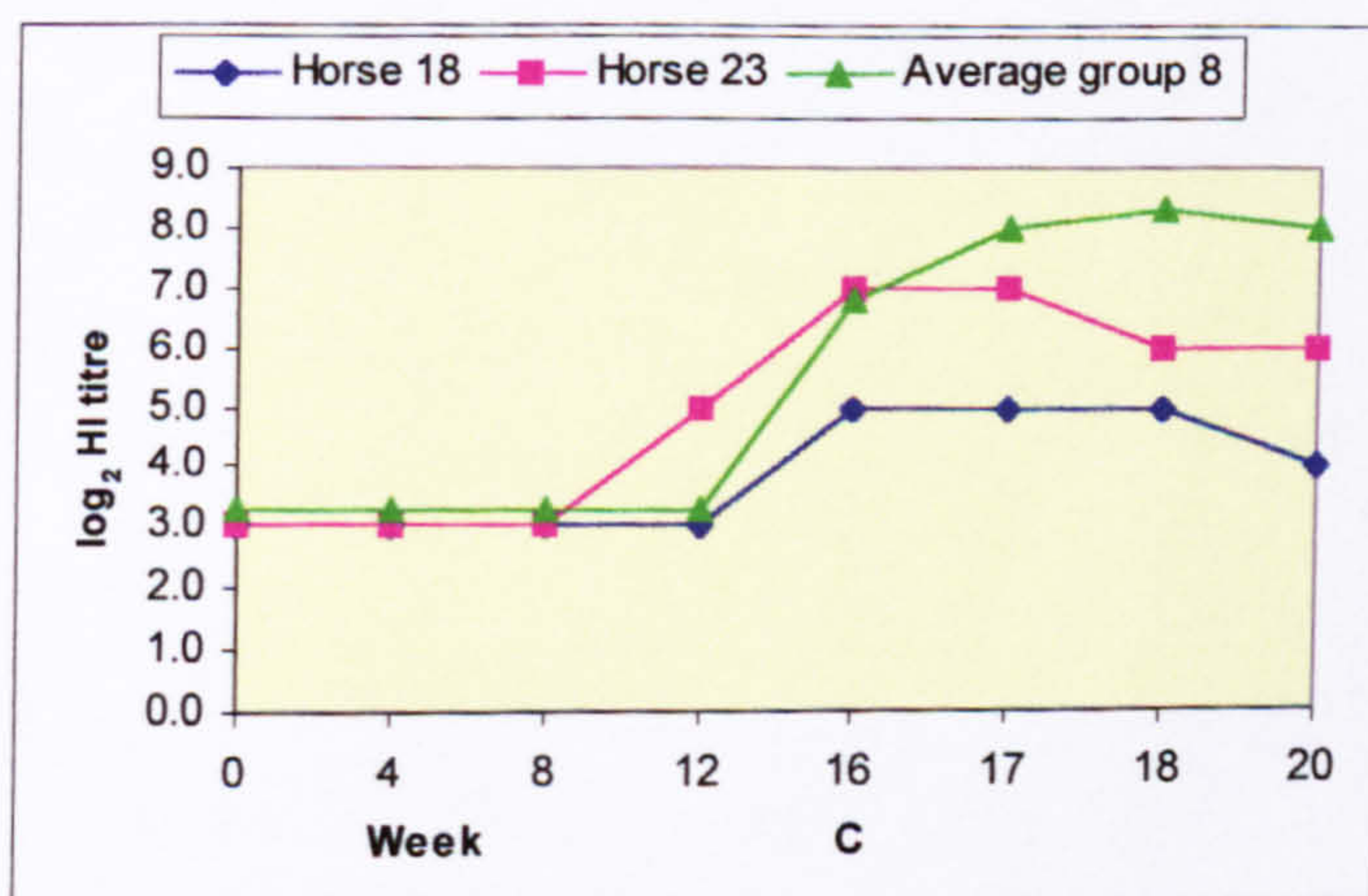


Figure 6.7 Comparison of HI titres of two of the best performing horses gives DNA vaccines with the average of the group vaccinated with classical vaccine.
C – time of challenge.

Table 6.4 Comparative serology at t = 16 weeks.

Group	Vaccine	Animal	Serological procedures		
			HI assay (log2 titre)	ELISA (log2 titre)	Immunofluor. (1/5 serum dilution)
1	HA+NP	30	4	< 1.6	-
		18	5	6.3	+/-
		21	3	4.7	+/-
		26	3	< 1.6	-
2	HA+NP (Diluvac Forte)	2	3	< 1.6	-
		22	3	< 1.6	-
		28	3	< 1.6	-
3	HA+NP+IFN γ	12	4	4.7	+
		15	3	4.7	+
		23	7	9.5	+
		24	3	4.7	+/-
4	HA+NP+IFN γ (Intramuscular)	9	3	< 1.6	-
		11	3	< 1.6	-
		16	6	6.3	+/-
5	HA+NP+IL18	5	4	6.3	+
		14	3	< 1.6	-
		27	3	< 1.6	-
		29	3	< 1.6	-
6	HA+NP+IL12+IL18	8	3	3.1	+
		10	3	3.1	+/-
		13	3	6.3	+/-
		20	3	< 1.6	-
7	empty vector	4	3	< 1.6	-
		17	3	< 1.6	-
		19	3	< 1.6	-
		1	3	< 1.6	-
8	classical vaccine	3	7	11.1	+
		6	6	9.5	+
		7	7	9.5	+
		25	7	6.3	+

6.4 Discussion

The poor pre-challenge production of antibodies in the great majority of horses in response to DNA vaccination and the higher HI antibody titres present after challenge, suggests that the level of immunity produced was not sufficient to completely prevent challenge virus replication. In contrast, vaccination of horses 16, 18 and 23 induced levels of antibodies before challenge, similar to those of the group vaccinated with classical vaccine. Additionally, and only in the case of horses 18 and 23, the titres remained constant following homologous challenge.

However the challenge virus failed to promote infection, as assessed both by the observation of clinical signs and by virus isolation from nasal secretions. This could be due to the immune status of the trial horses, improper dosing or alteration of the challenge inoculum after assessment of infectivity.

Consequently, the antibody titres pre and post-challenge could not be correlated with protection, or lack of it. Nevertheless, it has been reported that the elicitation of high HI titres following DNA vaccination against influenza is not an absolute requirement for protection to challenge (Fynan *et al*, 1993) and that the efficient priming of both B and Th cell memory by DNA vaccination is the underlying mechanism of protection. It is therefore particularly unfortunate that the challenge failed in this trial, as protection against challenge was the indicator for the development of protective immunity following vaccination.

The fact that only horses 16, 18 and 23 developed pre-challenge antibody titre comparable to that of animals receiving classical vaccine might be related to low efficiency of DNA transfer by the system used. Additionally, the use of the adjuvant Diluvac Forte (group 2), that nonspecifically boosts immune responses, and the use of intramuscular injection (group 4) as an alternative route (reportedly more efficient in the transfection of DNA *in vivo*) did not have an effect in the immune response to the vaccines when compared to results of groups 1 and 3, respectively. Therefore, the use of a more reliable means of DNA transfer (e.g. gene gun) would verify if the

responses observed in horses 16, 18 and 23 are due to casual increased DNA uptake or simply reflect an increased immune responsiveness of these horses.

The choice of cytokines co-delivered (IFN- γ , IL-12 and IL-18) was intended to shift the immune response to a Th1 setting. Th1 responses not only promote cell mediated immunity but also skew antibody production, disfavoring neutralising antibodies. The latter is suggested by results obtained for group 3, where IFN- γ was co-administered and also for animals 5 and 8 (receiving IL-18 and IL-12 + IL-18 co-administration, respectively). The serum of all these animals was able to significantly bind antigen both in ELISA and expressed by infected MDCK cells, despite the poor inhibition of haemagglutination, suggesting that at least some of the specific antibodies produced were not neutralising. In this context, it would be interesting to subtype the IgG response in the animals of group 3 and compare it to that of groups 1 and 8, investigating if there was a shift towards the equine equivalent of murine IgG2a subisotype. This would be in line with the observation that natural infection in horses gives rise to serum IgGa and IgGb protective responses (that mediate complement activation and ADCC in the horse) whereas conventional vaccination serum responses are restricted to IgG(T) (Nelson et al, 1998).

The hypothesis that these cytokines had operated a transformation in the type of immune response would be confirmed if horses of group 3, 5 and 6 were found to have increased CTL responses in comparison to those of group 1 (receiving the same vaccine without cytokines) and especially to those receiving classical vaccine (group 8) known to induce mostly humoral responses. Unfortunately the elicitation of cell mediated responses was not investigated and therefore this conjecture remains unconfirmed.

In summary, the results obtained, particularly for horses in group 3, suggest that there is some promise that co-administration of plasmids encoding HA, NP and Th1 cytokines would result in protection against influenza virus infection. If the trial is to be repeated, the following considerations should be addressed. Limiting the number of experimental groups and increasing the number of animals per group would result

in an increased statistical relevance of the results. The use of a more reliable method of delivery (e.g. gene gun) would address plasmid DNA transfer efficiency. The optimisation of the challenge material would allow proper assessment of protection and its correlation with antibody titres. The detection and measurement of CTL activity or at the very least, the isolation of T cells and investigation of their ability to proliferate in the presence of viral antigens, would facilitate the dissection of cytokine activity and protection mechanisms. Finally, the challenge of vaccinated animals with an heterologous influenza virus strain would assess the potential of cross protection attributed to these vaccines.

On a study published after completion of the present trial, Lunn et al (1999) report that DNA vaccination of ponies with the equine influenza hemagglutinin gene resulted in partial to complete protection from homologous challenge infection, which was accompanied by a reduction in challenge virus shedding. Additionally, serology of responsive horses showed a marked increase in antibodies of the IgGa and IgGb isotypes with no detectable IgG(T) responses. The authors conclude that "DNA vaccination has the potential to protect horses against influenza virus infection".

CHAPTER 7

GENERAL DISCUSSION

7.1 Introduction

This project had two main objectives. Firstly, the recombinant expression of equine interferon gamma and the development of biological assays suitable for the evaluation of equine interferon gamma activity *in vitro*. These objectives were largely attained with successful expression of equine interferon gamma in a variety of heterologous systems (*E. coli*, insect and mammalian cells), the production and characterisation of antibodies elicited against the recombinant protein and evidence for activity of the expressed protein in a diversity of assays based on well characterised cellular responses to interferon gamma exposure (chapter 3). The second objective was to clone additional equine Th1 type cytokines, i.e., interleukin-12 and interleukin-18 (chapter 4) and use them in a naked DNA vaccination trial against equine influenza virus (chapter 6), by co-administration with the cloned viral genes haemagglutinin and nucleoprotein (chapter 5). These experiments were devised not only to assess the feasibility of delivering equine cytokine genes *in vivo* but also to investigate the potential of the cloned genes to be used as modulators of immune responses to vaccination. Equine interleukins 12 and 18, as well as the nucleoprotein and haemagglutinin genes of equine influenza virus, were successfully cloned. Additionally expression of the cloned viral genes was demonstrated by transient plasmid transfection of mammalian cells. The results of the vaccination trial were mostly inconclusive, due to failure of the challenge and to the inadequacy of the process used for validation of the immune responses to vaccination. Nevertheless, the implementation of the trial made available considerable expertise, in the group, for the development and use of constructs for DNA immunisation. In addition, the data generated will be invaluable for future optimisation of trial specifications and efficacy assessment.

In summary the present project has provided several equine specific cytokine reagents, the development of a number of molecular and cell biology techniques and a model for the elicitation of virus specific immune responses in the horse. These tools will be useful in the study of the equine immune system as a whole and of the response to intracellular pathogens in particular. The applicability of these and other cytokine reagents is discussed below.

7.2 Cytokines in equine immunology

The use of non species specific cytokine reagents in veterinary medicine is often met with limited success, due to the low interspecies homology which translates as a lack of cross reactivity of these molecules between species. The cloning and expression of equine cytokine genes and downstream production of reagents will therefore be extremely useful to dissect equine immune responses in health and disease and to understand how cytokines influence such processes. Examples of reagents commonly used in cytokine research are cDNAs used for the production of primers and probes for mRNA quantitation by northern blot or real time PCR, polyclonal and monoclonal antibodies for the quantitation of protein by western blot or flow cytometry and responsive cell lines for the implementation of bioassays that measure biological potency.

The study of cytokine expression patterns and their functions is paramount to our understanding of the proper function of a healthy immune system and the basis for any attempt to influence disease onset and progression. From an evolutionary perspective it makes sense to predict that immunological effector and regulatory mechanisms established for one species are likely to be found in other species. In the case of equine medicine, studies performed in other ungulates and even primates are, in principle, more relevant than studies derived from more distant species, like rodents. Nevertheless, it is necessary to verify, rather than assume, the validity of key immunological concepts on an individual species basis.

Very little is known about the involvement of cytokines in equine immune responses apart from the well characterised influence of TNF- α , IL-1 and IL-6 in the pathogenesis of equine septic shock (Hannant D, 1998). The emergence of equine cytokine reagents will help elucidate questions that remain unanswered but for which there is seminal evidence. Is there a Th1/Th2 dichotomy in equine immune responses and is it possible to polarise equine T cell clones *in vitro* to one or the other phenotype? There is some evidence that vaccination of ponies against the metazoan parasite *Strongylus vulgaris* has different outcomes following challenge, depending

on the form and route of administration. While animals vaccinated by oral administration of radiation-attenuated *S. vulgaris* larvae were protected from challenge infection and displayed eosinophilia, characteristic of Th2 responses, parenteral administration of purified *S. vulgaris* antigens induced post challenge exacerbation of clinical signs with no detectable eosinophilia (Monahan et al, 1994). Additionally, as mentioned before, recovery from equine influenza infection is associated with the elicitation of complement fixing antibodies and cell mediated immune responses to viral antigens in vitro (Hannant and Mumford, 1989), which is consistent with a Th type 1 response. Are cytokines responsible for isotype class switching in antibody production in the horse? The fact that horses produce different isotypes of antibodies against influenza virus following vaccination or infection is an indication that the equine immune system is able to direct class switching. Whether this phenomenon is influenced by cytokines remains to be proven. We can now also begin to address other questions. Are equine cytokines involved in T cell regulatory mechanisms? Are there any pathologies of the horse associated with an incorrect bias in the T helper, T suppressor and/or B cell repertoires?

7.3 Equine cytokines for diagnosis and therapy

Cytokine production is in most cases a short-lived event, reflecting the activation status of the producing cell in response to microenvironmental stimuli triggered by immune aggression. Cytokines tend to have a short radius of action and are active in minute amounts on the surface of target cells. It is this space and time restriction that enables cytokines to shift local immune responses influencing the type of protective effector mechanisms elicited without compromising subsequent regulation by antagonistic signals. However, this same constraint constitutes a major difficulty in the analysis of cytokine expression. If we are to correlate cytokine profiling with protective versus non-protective immunity or with disease susceptibility, it is important to correlate levels of cytokine mRNA expression with actual protein output and often with evidence of cellular activity attributable to stimulation by the measured cytokines. Studies like this will increase our understanding of cytokine functional synergism, overlap and antagonism, narrowing the concept of redundancy in cytokine

activity. This in turn will be of value in the diagnosis and prognosis of disease processes and in the design and application of immune-therapy regimens.

The main drive for cytokine research in veterinary medicine is their potential for the development of new and better drugs. Cytokines and cytokine reagents can not only be used as drugs themselves but also to assess the efficacy of immuno-stimulatory compounds (e.g. vaccine adjuvants, steroids, antiviral drugs) in the treatment or prevention of disease. The use of recombinant cytokines as therapeutical agents for ungulates has been very limited and non-existent in the case of the horse. Bovine IL-2 (rBoIL-2) reduced severity of clinical disease in vaccinated calves challenged with bovine herpes virus (BHV)-1 (Reddy et al, 1989). BHV infected cattle treated with both IFN- α and IFN- γ had a marked reduction in the severity of illness and mortality (Babiuk et al, 1991). Also BoIFN- γ was found to reduce the severity of pneumonia caused by *Haemophilus somnus* (Peel et al, 1990) and the degree of septicaemia due to *Salmonella typhimurium* (Chiang et al, 1990). BoIFN- γ , IL-1 β and IL-2 have also been used in the control of mastitis, having promoted lower infection rates (Daley et al, 1991).

The delivery of recombinant cytokines *in vivo*, usually produced in bacterial expression systems, has certain disadvantages. It promotes variable levels of systemic toxicity that can modify or dilute the activity of the administered cytokine. It can elicit immune responses, in the form of antibodies, to the inoculated cytokine, therefore rendering it inactive. These proteins are produced in heterologous expression systems and as such post-translation modifications are not always authentic. This has a bearing in the biological activity, immunogenicity and pharmacokinetics of the inoculated cytokine. Recent developments in minimal viral and bacterial vectors and DNA vaccination technologies allow cytokine genes to be delivered and expressed *in vivo*, providing an effective and inexpensive commercial delivery method via feed, water or aerosol. Such systems allow cytokines to be produced in their native form, as delivered cytokine genes are transcribed and translated using the host cellular enzymatic machinery. Cytokines like IL-2, IL-4, IFN- γ and IL-12 delivered by these systems in murine models have proven to be potent immunomodulators, particularly

when used as adjuvants for vaccine formulations (Braciak et al, 1993; Kurilla et al, 1993; York et al, 1996; Cohen et al 1998; reviewed in chapter 6) and are held as a promising resource in the fight against cancer and infectious diseases.

7.4 Future directions

Future work in equine cytokine research will have to pass through the development of more and better reagents. Of great importance is the establishment of equine cell lines, particularly of haematopoietic origin and of neutralising monoclonal antibodies that will facilitate the characterisation of native cytokines and the assessment of biological activity of recombinant forms of these proteins. The manipulation of cytokine expression *in vivo* will benefit from the cloning of soluble forms of cytokine receptors for specific blocking of particular cytokines, and from tissue-targeted delivery of cytokines and anti-cytokines. This can be achieved either by using vectors with specific tissue tropism (e.g. containing the adenovirus fiber gene) or containing host tissue-specific promoters.

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APPENDIX-I

Short Communication

Cloning and Sequencing of Equine Transforming Growth Factor-Beta 1 (TGF β -1) cDNA

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Transforming growth factor beta (TGF- β) belongs to a family of peptide growth factors which control critical stages of cell proliferation and differentiation. We report the cloning and sequencing of the cDNA for TGF- β type 1 isoform of the horse. The predicted mature equine TGF β -1 peptide is 112 amino acids in length and exhibits 99% identity to mature human TGF β -1.

Keywords: Cytokine, molecular cloning, polymerase chain reaction, sequencing, transforming growth factor

Transforming growth factor beta (TGF- β) is a ubiquitous and multifunctional cytokine produced by normal and transformed tissues. Its biological activities include modulation of cell growth and differentiation and a role in immune and inflammatory responses (reviewed in Derynck, 1994). TGF- β is present in high concentration in platelets (Childs *et al.*, 1982), kidney (Roberts *et al.*, 1983), placenta (Frolik, 1983) bone and spleen (Roberts and Sporn, 1992) and is also released in significant amounts by activated lymphocytes and macrophages (Kehrl *et al.*, 1986). Three isoforms of this cytokine (TGF- β 1, TGF- β 2

and TGF- β 3) have been identified in mammalian species (Derynck, 1994). A wide range of biological activities have been associated with TGF β s *in vivo* including involvement in processes as distinct as embryogenesis, tissue repair, wound healing, immunosuppression and carcinogenesis (Roberts and Sporn, 1990; Wahl, 1994). TGF β may have clinical application in immunosuppressive regimes, as a therapeutic agent in wound and fracture healing and in the treatment of acute inflammatory processes (Sporn *et al.*, 1986).

Human TGF- β 1 is synthesised as a precursor polypeptide of 390 amino acids, containing an N-terminal signal peptide, a pro-segment (the latency-associated polypeptide, LAP) and a 112 amino acid C-terminal peptide (mature TGF- β monomer).

TGF- β is usually secreted as an inactive complex composed of two pro segments noncovalently associated with a covalently linked homodimer of the C-terminal peptide. The biologically active form is a 25kDa homodimer of the

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1 ATCCCGCCCTCCGGCCTGCGGCTGCTGCCGCTGCTGCTGCCACTGCTGTGGCTACTAGTG
 M P P S G L R L L P L L L P L L W L L V
 SP↑
 61 CTGACGCCTGGCCGGCCAGCCGCCGACTGTCCACCTGCAAGACCATCGACATGGAGCTG
 L T P G R P A A G L S T C K T I D M E L
 * LAP↑
 121 GTGAAGCGGAAGCGCATCGAGGCCATCCGCGGCCAGATCCTGTCCAAGTTGCGGCTCGCC
 V K R K R I E A I R G Q I L S K L R L A
 181 AGCCCCCGAGCCAGGGGGAGGTTCCGCCCCGGCCGCTGCCCGAGGCCGTGCTGGCCCTT
 S P P S Q G E V P P G P L P E A V L A L
 241 TACAACAGTACCCGCGCCAGGTGGCCGGGAGAGCGCTGAGACGGAGCCCGAGCCTGAG
 Y N S T R A Q V A G E S A E T E P E P E
 * * *
 301 GCGGACTACTACGCCAAGGAGGTCACCCGCGTGCTAATGGTGAAAAGGAAAACGAAATC
 A D Y Y A K E V T R V L M V E K E N E I
 * *
 361 TATAAGACTGTGGAGACCGGCTCACACAGCATATATATGTTCTTCAACACGTCGGAGCTC
 Y K T V E T G S H S I Y M F F N T S E L
 * * * * *
 421 CGGGCAGCAGTGCCCGATCCCATGCTGCTCTCCCGGGCAGAGCTGCGCCTCCTAAGGCTC
 R A A V P D P M L L S R A E L R L L R L
 * * *
 481 AAGTTAAGCGTGGAGCAGCATGTGGAGCTGTACCAGAAATACAGCAATAATTCCTGGCGC
 K L S V E Q H V E L Y Q K Y S N N S W R
 *
 541 TACCTCAGTAACCGGCTGCTGACCCCCAGCGACTCGCCGGAATGGCTGTCCTTTGATGTC
 Y L S N R L L T P S D S P E W L S F D V
 *
 601 ACTGGAGTCGTGCGGCAGTGGCTGAGCCAGGGAGGGGCAATGGAGGGCTTTCGCCTCAGT
 T G V V R Q W L S Q G G A M E G F R L S
 * * *
 661 GCCCACTGCTCCTGTGACAGCAAAGATAACACACTCCGCGTGGGCATCAACGGGTTCAGT
 A H C S C D S K D N T L R V G I N G F S
 * * *
 721 TCCAGTCGCCGGGTGATCTGGCCACCATTGATGGCATGAACCGGCCCTTCCTGCTCCTC
 S S R R G D L A T I D G M N R P F L L L
 * * *
 781 ATGGCCACCCCACTGGAGAGGGGCCAGCAGCTGCACAGCTCCCGGCACCGCCGAGCTCTG
 M A T P L E R A Q Q L H S S R H R R A L
 * * MP↑
 841 GACACCAACTACTGTTTCAGCTCCACAGAGAAGAACTGCTGCGTACGGCAGCTGTACATT
 D T N Y C F S S T E K N C C V R Q L Y I
 901 GACTTTCGCAAGGATCTGGGCTGGAAGTGGATCCACGAGCCCAAGGGCTACCACGCCAAC
 D F R K D L G W K W I H E P K G Y H A N
 961 TTCTGCCTGGGGCCCTGCCCCCTACATTGAGCCTGGACACGCAGTACAGCAAGGTCCTG
 F C L G P C P Y I W S L D T Q Y S K V L
 1021 GCCCTGTACAACCAGCACAACCCGGGCGCGTGGCGGGCGCGCTGCTGCGTGCCGCAGGTG
 A L Y N Q H N P G A S A A P C C V P Q V
 *
 1081 CTGGAGCCGCTGCCCATCGTGTACTACGTGGGTGCGAAGCCCAAGGTGGAGCAGCTGTCC
 L E P L P I V Y Y V G R K P K V E Q L S
 1141 AACATGATCGTGCGCTCCTGCAAGTGCAGCTGA 1173
 N M I V R S C K C S *

FIGURE 1 Nucleotide sequence of the equine TGFβ-1 cDNA and predicted amino acid sequence of its product. Primers flanking the entire open reading frame were 5'- TTAGATATCGCCTCCCCCATG-3' and 5'- TCAGCTGCACTTGCAGGAG -3'. PCR reaction mix was: 1 × buffer, 200μM dNTP, 2mM MgCl₂, 2 units of Taq polymerase, 50pmol of each primer and 10μl of cDNA. Thermal cycling conditions were 94°C 5min, then 35 cycles of 94°C 30sec, 45°C 45sec, 72°C 2min, terminating with a single cycle of 72°C for 7min. Primer determined nucleotide sequence and potential N-linked glycosylation sites are underlined. Amino acids which differ from those of human TGFβ-1 (Derynck *et al.*, 1985) are asterisked (*). Arrows indicate predicted first amino acid in signal peptide (SP↑), latency-associated polypeptide (LAP↑) and mature peptide (MP↑).

mature C-terminal peptide (Assoian *et al.*, 1983) which is highly conserved (>99%) in amino acid sequence cross species. To date the sequences of TGF- β 1 cDNAs or genes isolated from more than ten species, including human (Derynck *et al.*, 1985), bovine (Obberghen-Schilling *et al.*, 1987), porcine (Dernyck *et al.*, 1987) and canine (Manning *et al.*, 1995), have been published or deposited in databases.

In order to isolate equine TGF- β 1 cDNA, lymphocytes were recovered from spleen and lymph node and stimulated for six hours with concanavalin A (7.5 μ g/ml) or lipopolysaccharide (10 μ g/ml). mRNA was isolated using "Quick prep mRNA isolation Kit" (Pharmacia) and subsequently treated with methylmercury hydroxide (10mM). cDNA synthesised using "First Strand cDNA Synthesis Kit" (Pharmacia) primed with an oligo-dT, served as template for amplification using Taq based Perkin Elmer PCR System and a series of primers derived from published human and bovine TGF- β 1 sequences (Derynck *et al.*, 1985; Obberghen-Schilling *et al.*, 1987). PCR products were cloned into TA Cloning Vector (Invitrogen). A total of thirty five clones from eight independent PCR reactions were sequenced by automated sequencing using "ThermoSequenase Cycle Sequencing Kit" (Amersham) and M13 forward and reverse primers labelled with IRD41. GCG programs were used to analyse sequencing data and to generate the consensus sequence shown in Figure 1.

Analysis of the sequence data revealed an open reading frame encoding a 390 amino acid equine TGF- β 1 precursor which possesses the following features conserved throughout all TGF- β 1 precursors analysed to date—a region of hydrophobic amino acids (position 8–21) within the signal peptide (1–29), twelve cysteine residues (nine within the mature peptide), an arginine doublet (277–278) preceding the proteolytic cleavage site utilised in the generation of the mature peptide sequence (C-terminal 112 amino acids, 279–390) and a CXCX motif at the extreme C-terminus (Fig. 1).

The nucleotide sequence and derived amino acid sequence exhibit homology in the range 90–92% and 89–92%, respectively, with other published mammalian TGF- β 1 sequences. Within the predicted mature region the equine sequence differs from the human at one position. Equine TGF- β 1 is thus the only characterised non-rodent mammalian TGF- β 1 which is not 100% conserved in sequence to human TGF- β 1 within the biologically active peptide.

Cloning of equine TGF- β 1 cDNA will facilitate the study of the role of this cytokine in the pathogenesis of equine diseases. For instance TGF- β 1 has been implicated in the fibrosis associated with chronic pneumonia in the horse (Rodriguez *et al.*, 1996) and abnormal expression of this cytokine may be associated with the pathogenesis of osteochondrosis in the horse since, along with insulin like growth factor II, TGF- β 1 is required for endochondral ossification. (Welin, 1993). Furthermore the expression of recombinant TGF- β 1 will permit the evaluation of the therapeutic role of this cytokine in equine medicine particularly in the treatment of chronic soft tissue wounds.

Acknowledgements

The nucleotide sequence data reported in this paper will be shared by the EMBL, GenBank, DDBJ, NBRF-PIR, MIPS and JIPID databases with the accession number: X99438.

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Short Communication

Nucleotide Sequence of Equine Caspase-1 cDNA

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Caspases are a family of cysteine proteases which have important roles in activation of cytokines and in apoptosis. Caspase-1, or interleukin-1 β converting enzyme (ICE), promotes maturation of interleukin-1 β (IL-1 β) and interleukin-18 (IL-18) by proteolytic cleavage of precursor forms to generate biologically active peptides. We report the cloning and sequencing of equine caspase-1 cDNA. Equine caspase-1 is 405 amino acids in length and has 72% and 63% identity to human and mouse caspase-1, respectively, at the amino acid level. Sites of proteolytic cleavage and catalytic activity as identified in human caspase-1, are conserved.

Keywords: caspase, interleukin-1 β , equine, protease, sequence

Caspases are a family of more than ten cysteine proteases which play an important role in inflammatory processes and in apoptosis (Wong, 1998). Family members are characterised by a specificity for aspartic acid in the P₁ position and a conserved QACXG active site pentapeptide region (X=Q, R or G) (Cohen, 1997). Interleukin-1 β converting enzyme (ICE), or caspase-1, was the first discovered member of the family (Thornberry et al., 1992). Caspase-1 processes

prointerleukin-1 β from its inactive 31kDa form to the bioactive 17kDa cytokine IL-1 β which is a key mediator of inflammation (Black et al., 1989). More recently a role in the maturation of interleukin 18 (IL-18, previously known as interferon- γ inducing factor, IGIF) has been identified (Gu et al., 1997; Ghayur et al., 1997). Caspase-1 cleaves inactive 24kDa proIL-18 to release a bioactive 18.2kDa peptide which has a critical role in the development of a Th 1 type cell-mediated immune response (Okamura et al., 1995). Immunoelectron microscopic staining techniques suggest that active caspase is localised on the plasma membrane of cells, while proenzyme is mainly found in the cytoplasm (Ayala et al., 1994, Singer et al., 1995) and it has therefore been postulated that caspase-1 may be part of a transmembrane complex required for IL-1 β , and possibly IL-18, secretion (Tocci, 1997).

A role for caspase-1 in apoptosis was inferred due to sequence homology with the *C. elegans* cell death gene (*ced-3*). This was supported by demonstration of apoptosis in cells transfected

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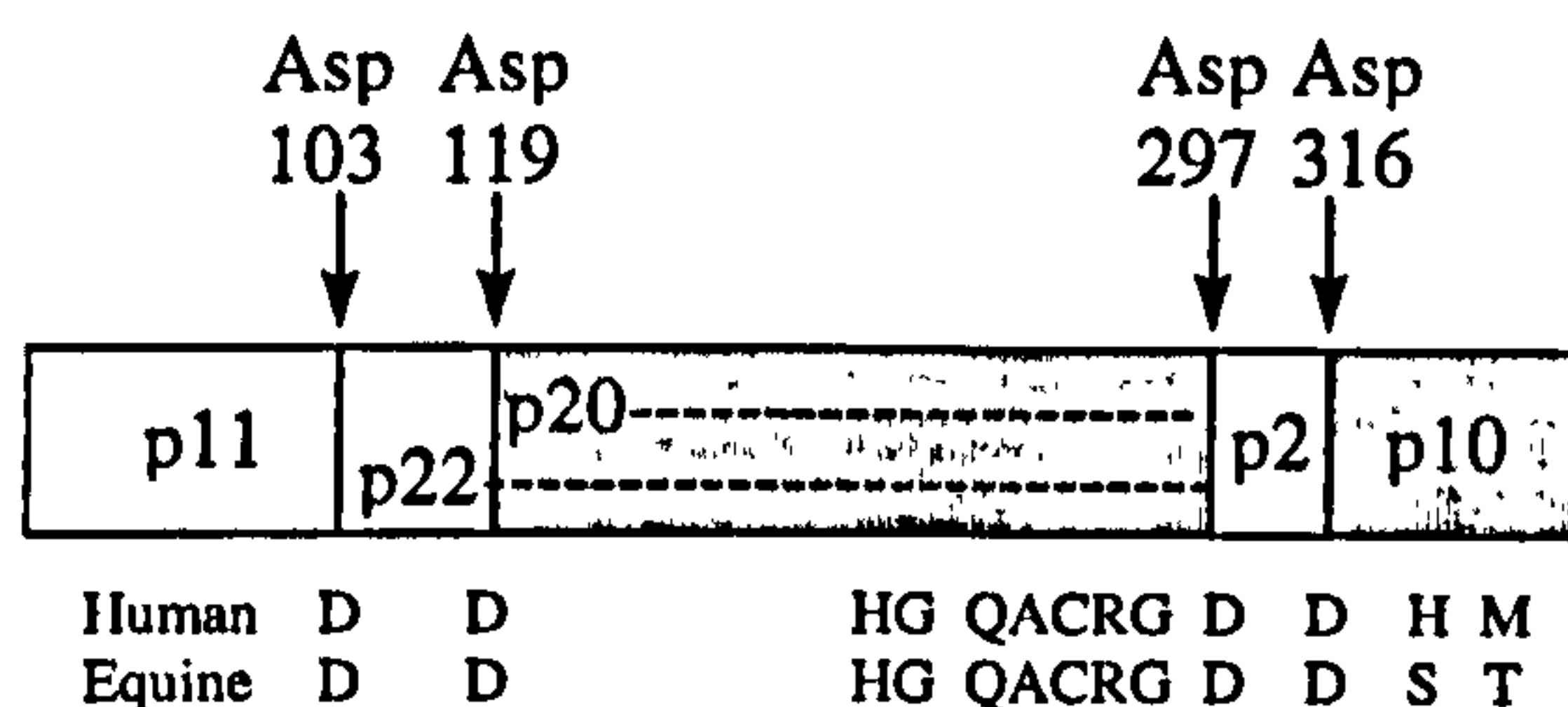


FIGURE 1 Schematic diagram of human caspase-1 (adapted from Tocci, 1997). Human caspase-1 amino acid residues involved in autoproteolytic cleavage, catalytic activity and binding of substrates or inhibitors are indicated and the corresponding residue in equine caspase-1 detailed below (see text for details)

with caspase-1 and by inhibition of this activity through mutation of the catalytic site (Miura *et al.*, 1993; Yuan *et al.*, 1993). However, the mechanism of apoptotic induction by caspase-1 is indirect and its physiological importance is unknown and it appears that other cysteine proteases, such as caspase-3, may play a more critical role in apoptosis (Miller, 1997; Nicholson *et al.*, 1995).

The functional human caspase-1 enzyme comprises two heterodimers composed of 20kD and 10kDa subunits derived from a 45kDa proenzyme by autoproteolytic cleavage at 4 aspartate (Asp) residues at positions 103, 119, 297 and 316 (Thornberry *et al.*, 1992; Ramage *et al.*, 1995; Tocci, 1997) [Figure 1].

Nucleotide sequences are available for a number of mammalian caspase-1 cDNAs including human (Thornberry *et al.*, 1992), mouse (Casano *et al.*, 1994) and rat (Kaushal *et al.*, 1998). An RT-PCR strategy using primers derived from conserved regions of these sequences was used to amplify equine caspase-1 cDNA. Cells recovered from equine lung washes were stimulated with concanavalin A (7.5µg/ml) or lipopolysaccharide (10µg/ml) and mRNA isolated (Quick prep mRNA isolation kit, Pharmacia Biotech). First strand cDNA (First Strand Synthesis kit, Pharmacia Biotech) was used as an

initial template in a 2-round PCR protocol [see legend Figure 2]. PCR products were cloned into pcDNA3.1/V5/His-TOPO mammalian expression vector (Invitrogen) and sequencing performed using a Thermosequenase Cycle Sequencing Kit (Amersham), IRD-800 modified primers (MWG Biotech) and a LICOR-4000 automated sequencer. Consensus sequence was derived from analysis of both strands of 10 equine caspase-1 cDNA clones isolated by PCR of templates derived from 6 independent primary PCRs. Three positions of allelic difference were detected at which 30% to 70% of clones possessed one of two nucleotides [Table I].

The 1218bp consensus equine caspase-1 sequence [Figure 2] encodes a predicted 405 amino acid polypeptide, one amino acid more than full length human caspase-1. No attempt was made to determine whether isoforms of equine caspase exist analogous to those identified for human caspase-1. The three allele-specific bases at positions 555, 607 and 914 [Table I, Figure 2] result in conservative differences or no amino acid difference. The predicted sequences have 82%/72% identity with human caspase-1, 74%/63% identity with mouse caspase-1 and 73%/64% with rat at the nucleotide/ amino acid level respectively.

Equine caspase-1 contains the conserved QACRG active site pentapeptide in the predicted p20 subunit (Cohen, 1997). Catalytic site residues Cys²⁸⁵, His²³⁷ and G²³⁸ (Walker *et al.*, 1994; Wilson *et al.*, 1994) are conserved along with those forming the carboxylate binding domain in human caspase-1 (Arg¹⁷⁹, Arg³⁴¹, Gln²⁸³ and Ser³⁴⁷). The four Asp residues associated with autoproteolytic cleavage of human caspase-1 are conserved in equine caspase-1, therefore it would be reasonable to predict that these proteins have closely related processing pathways and structural features. Divergence at potential inhibitor binding residues, His to Ser at 342 (equine 343) and Met to Thr at 345 (equine 346) [Figure 1], has been observed in other members of the caspase-1 (ICE) family (Miller *et al.*, 1997).


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atggccgacaaggtcctgaaggAAAAGAGGAGGCTGTTTATCCGCTCAGTGGGCACGGGT 60
M A D K V L K E K R R L F I R S V G T G

ACAGTAAATAGCTTGCTGGATGAACTGTTAGAGAAAAGGGTGCTGAACCAGGAAGAGATG 120
T V N S L L D E L L E K R V L N Q E E M

GAGAAAGTAAGAGATGAAATGCTACAGTTATGGATAAGGCCCGAGCTTTGATTGATGCT 180
E K V R D E N A T V M D K A R A L I D A

GTCATTTCGGAAAGGGCCCCAGGCATGCCAAATTTTATCGGTCACATTTGTGAAGATGAC 240
V I R K G P Q A C Q I F I G H I C E D D

CCCCACCTTGCAGAGACACTGCGCCTATCCTCAGGTCCACAATCTGGAAATTTTCTTAAA 300
P H L A E T L R L S S G P Q S G N F L K

ACACAAGACTCCCAAGCAGTGGTTCATTCCTCCCCAGCTCTTCAGGCAATGCCGGATGAC 360
T Q D S Q A V V H S S P A L Q A M P D D

CTGGCTAAGCTTGCATTGTCAGGGCCAAAAGTGAGCCTCAAGCTTTGTTCCACCAGAAGTG 420
L A K L A L S G P K V S L K L C S P E V

GTCGAAAGGATATGGAAGAAAAGTCAGCAGAGATGTACCCAATAATGGGAAAGTCGATG 480
V E R I W K E K S A E M Y P I M G K S M

ACCCGCACACGGCTTGCCTCATTATCTGCAACACAGAGTTTGACAATCTTCCAGGAGA 540
T R T R L A L I I C N T E F D N L S R R
*
GCTGGAGCTGAAGTTGATATTGCAAGTATGAAGGTGCTGCTAGAAGGTCTGGGGTACAGT 600
A G A E V D I A S M K V L L E G L G Y S

GTGGAAGTGAAAGAAAATCTCACCGCTTTGGACATGACTACAGAGCTGAAGGCATTTGCT 660
V E V K E N L T A L D M T T E L K A F A

GCCCCGCCAGAGCACCGTCTCTGACAGCACTTTCCTGGTGTTCATGTCTCATGGTATT 720
A R P E H R S S D S T F L V F M S H G I

CGAGAAGGCATTTGTGGGAAGAAATTCTCTGAGAAAGTCCCAGATGTATTAGAAGTCAAC 780
R E G I C G K K F S E K V P D V L E V N

ACCATCTTTCAAATTTTAAACACCAGGAAGTGTCAAATTTGAGGGACAAACCCAAGGTG 840
T I F Q I F N T R N C P N L R D K P K V

ATCATTATCCAGGCCTGCCGTGGTGAGAATCAAGGGGTAGTGTGGTTAAAAGACTCAACA 900
I I I Q A C R G E N Q G V V W L K D S T

GGAACTTCTGGAATAGCTCCTCACTGGCTCCAGACGATTTTGAGGATGATGCCATTAAG 960
G T S G N S S S L A P D D F E D D A I K

AAAGCCCATGTAGAGAAAGATTTTATTGCTTTCTGCTCTTCAACACCAGATACTGTTTCT 1020
K A H V E K D F I A F C S S T P D T V S

TGGCGAAGTCCCACAACGGGCTCTGTTTTTCATTGAGAACTCATTGAAAATTTGCAAGAA 1080
W R S P T T G S V F I E K L I E N L Q E

TATGCCTGGTCTGTGATCTGGAGGAAATTTCCGAAAGGTCCGATTATCATTTGAGTTG 1140
Y A W S C D L E E I F R K V R L S F E L

CCAGATGCTAGGGCACAGATGCCCACTGCCGAAAGAGTGACTTTGACAAGACGCTTCTAC 1200
P D A R A Q M P T A E R V T L T R R F Y

ctcttcccaggacattaa 1218
L F P G H *

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FIGURE 2 Nucleotide sequence of equine caspase-1 and predicted amino acid sequence of its product. Primer-derived sequence is in lower case. The essential QACXG active site pentapeptide motif is underlined along with key residues associated with catalytic activity, autoproteolytic cleavage, and inhibitor binding – these are 1 position further from the N-terminus than the corresponding human caspase-1 residues as equine caspase-1 has an amino acid insertion at position 160. Positions of allelic variation are underlined. Primary PCRs were performed using 10µl cDNA template, 1 unit BioTaq (Bioline) and primers 5'-GATATCATGGCCGACAAGGTCTGAAGG-3' and 5'-GCGCCCCGGGTTTCCTTAGTTTAATG-3', in a 50µl reaction volume containing 1x Bioline core reagents. Secondary PCRs were performed using 0.5µl of primary PCR reaction product as template, 1 unit BioTaq or Pfu (Stratagene) and primers 5'-GGCGTTTAAACCCATGGCCGACAAG-3' and 5'-GCAGCTGTTAATGTCCTGGGAAGAG-3' in a 50µl reaction volume containing 1x Bioline core reagents or Stratagene core reagents. Thermocycling conditions were as follows: 95°C 5' min then for primary PCRs 30 cycles of 95°C 30s, 37°C 30s, 72°C 1'20s and for secondary PCRs 25 cycles of 95°C 30s, 45°C, 30s, 72°C 1'20s. The final extension time for both primary and secondary PCRs was 72°C for 7 min

TABLE I Allelic differences within equine caspase-1 coding sequence and effect on amino acid sequence of expressed peptides

Nucleotide position	Nucleotide	Amino acid position	Coding potential
555	A/T	185	no change
607	G/T	203	L/V neutral, nonpolar
914	A/G	305	S/N neutral, polar

Caspase-1 inhibitors may have potential in the treatment of inflammatory disease and in antiapoptotic regimes. Determination of the primary sequence of equine caspase-1 and production of expression cassettes will facilitate *in vitro* studies and ultimately an investigation of equine caspase-1 or inhibitors as prophylactic or therapeutic agents in the horse.

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BRIEF COMMUNICATION

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Cloning and sequencing of horse interleukin-12 and interleukin-18 cDNAs

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Interleukin-12 (IL-12) and interleukin-18 (IL-18) are key cytokines in the modulation of cell-mediated immune responses. IL-12 is produced primarily by macrophages and dendritic cells in response to intracellular parasites, bacteria, and their products (reviewed in Trinchieri 1995). First identified by its synergy with IL-2 in the stimulation of the activity of T cells (Gately et al. 1986) and natural killer (NK) cells (Kobayashi et al. 1989), it is now known that this molecule plays a central role in the modulation of immune responses. The effects of IL-12 include the enhancement of lytic activity and IFN- γ secretion by NK and T cells, the inhibition of IL-2-driven proliferation of NK cells, the potent proliferation induction of both naive and memory T cells, and the induction of T-helper type 1 (TH1) differentiation (Murphy et al. 1994; Trinchieri 1997). Biologically active human IL-12 is a 70 000 M_r heterodimer comprising covalently linked glycosylated peptides of approximately 40 000 and 35 000 M_r , designated p40 and p35 respectively (Trinchieri 1995). Sequence simi-

larity of p35 to IL-6 and granulocyte colony stimulating factor (G-CSF) (Merberg et al. 1992) and of p40 to the IL-6 and G-CSF receptors (Gearing and Cosman 1991) are suggestive of evolution from a common primordial cytokine and a p70 structure akin to a soluble cytokine:cytokine receptor complex.

Interleukin-18, originally termed interferon-gamma inducing factor (IGIF), is a cytokine functionally identified by Okamura and co-workers (1982) as a factor which induced interferon gamma production in mice with endotoxic shock. Primarily produced by macrophage-like cells, it is regarded as functionally similar to IL-12. Activities include the induction of IFN- γ and GM-CSF, repression of IL-10 production, stimulation of T-cell proliferation, and enhancement of NK cell cytotoxicity (reviewed in Kohno and Kurimoto 1998; Ushio et al. 1996). Human IL-18 is synthesized as a biologically inactive precursor protein, proIL-18, of 193 amino acids which is processed into an 18 000 M_r mature peptide devoid of N-linked glycosylation by proteolytic cleavage of an N-terminal 36 amino acid leader sequence. Cleavage is catalyzed by caspase-1 (interleukin-1 β converting enzyme, ICE) and associated events may facilitate export of the bioactive monomeric 18 000 M_r peptide (Ghayur et al. 1997; Gu et al. 1997). IL-18 shares no significant similarity at the primary sequence level to any other cytokines. However, analysis of the sequence using fold recognition algorithms has revealed a distant relationship to the interleukin-1 superfamily (Bazan et al. 1996). While IL-12 and IL-18 both induce IFN- γ , IL-18 is, in the presence of appropriate costimuli, a more potent inducer of IFN- γ than IL-12 (Kohno et al. 1997). Receptor binding and signalling pathways are distinct and IL-12 and IL-18 can act synergistically in the induction of IFN- γ , an effect which may be contributed to by the ability of IL-12 to up-regulate IL-18 receptor expression (Ahn et al. 1997; Robinson et al. 1997).

The potent IFN- γ induction of IL-12 and IL-18 and associated immune effects have been elicited in prophylactic and therapeutic regimes through exogenous

The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the accession numbers Y11129, Y11130, and Y11131

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administration of recombinant cytokines. With a view to evaluation of IL-18 and IL-12 as immune modulators in domestic animals we have cloned and sequenced the horse, dog [D.J. Argyle and co-workers (1999), and cat L. Hanlon and co-workers, unpublished data, accession number Y13923] IL-12, IL-18, and caspase-1 cDNAs (Wardlow et al. 1999; accession numbers AF090119, AF135967, and AF135968). We report here the nucleotide sequence of horse IL-12 and IL-18 cDNAs derived by reverse transcription-polymerase chain reaction (PCR) of mRNA isolated from adherent cells recovered from horse lung washes. Cell pellets were resuspended at 1×10^6 cells/ml in complete medium (RPMI 1640, 10 mM Hepes, 2 mM glutamine, 5×10^{-5} M β -mercaptoethanol, 2% fetal bovine serum, 100 I.U./ml penicillin/streptomycin) and adherent cells subsequently washed and incubated for 6 h with lipopolysaccharide (10 μ g/ml). Cells were lysed and mRNA isolated using a Quick Prep mRNA Isolation Kit (Amersham Pharmacia Biotech, Bucks, UK) followed by treatment with methylmercury hydroxide (10 mM). mRNA was reverse transcribed using a First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech) with oligo-dT as primer. Primers designed from homologous regions of other mammalian p35 and p40 cDNAs were used to amplify horse IL-12 cDNA by Perkin Elmer *Taq*-based PCR (Table 1) in a nested PCR strategy. PCR reaction mixes comprised template (10 μ l of cDNA for primary PCRs and 1 μ l of primary PCR product for secondary PCRs), 50 pmol of each primer, 200 μ M dNTP, 2 mM $MgCl_2$, and 2 units of *Taq* polymerase in $1 \times$ buffer. Thermal cycling conditions were 94°C for 5 min, then 30 cycles of 94°C:1 min, 37°C (primary PCRs) or 55°C (secondary PCRs):1.5 min and 72°C:2 min, terminating with a single cycle of 72°C for 7 min. Overlapping subfragments of each cDNA were generated as full-length p35 and p40 cDNAs could not be amplified: full-length p35 and p40 were subsequently derived by conventional cloning strategies. PCR products were cloned into TA cloning vector pCR3.1 (Invitrogen, Groningen, The Netherlands) and 15 p40 clones and 15 p35 clones were sequenced across both strands using a Thermosequase Cycle Sequencing Kit (Amersham Pharmacia Biotech), IRD-800 modified primers (MWG Biotech, Mil-

ton Keynes, UK) and a LICOR-4000 automated sequencer. Recovery of fragments representing the entire horse IL-18 cDNA sequence required a nested PCR strategy using primers A + B or A + D for primary PCR and A + B or C + D for secondary PCR (Table 1). Templates used were cDNA for primary PCRs and 1 μ l primary PCR reaction mix for secondary PCRs. Primary PCR conditions were 94°C for 45 s, 45°C:1 min, 72°C:1.5 min for 30 cycles, and secondary PCR conditions were 94°C:45 s, 45°C:1 min, 72°C:2 min for 30 cycles. PCR products were cloned into pCR3.1 and 10 clones sequenced across both strands. Full-length horse IL-18 cDNA was subsequently recovered by PCR using primer A and a horse-specific primer, 5'-GCTAGTTCTGGTTTTGAACA-3'. Template used was cDNA and the following thermocycling conditions were applied: 94°C:55 s, 47°C:40 s, 72°C:2 min for 30 cycles. Consensus sequences, generated using GCG software (Wisconsin package), were deduced from clones derived from multiple independent primary and secondary PCR reactions to exclude contribution by *Taq*-induced errors (Figs. 1–3).

The predicted horse p35 peptide is 222 amino acids in length with a putative N-terminal signal peptide sequence and four potential N-linked (N-X-S/T) glycosylation sites (Fig. 1). Comparison with human and mouse p35 peptides revealed identities of 82% and 59%, respectively. The predicted horse p40 peptide is 329 amino acids in length and features a putative signal peptide sequence and three potential N-linked glycosylation sites (Fig. 2). A presumed allelic difference occurs at base 714 (A or G), as clones from several independent PCR reactions varied in sequence at this position. The amino acid encoded is unaffected by the substitution. Identities of 86% and 66% exist with human and mouse p40 peptides, respectively. The horse IL-18 cDNA possesses an open reading frame of 582 base pairs encoding a 193 amino acid protein lacking N-linked glycosylation sites (N-X-S/T) (Fig. 3). Identity of the predicted sequence to human and mouse IL-18 cDNAs is 87% and 77% at the nucleotide level and 79% and 67% at the amino acid level, respectively. Production of bioactive human IL-18 is associated with cleavage of full-length proIL-18 between Asp₃₆ and

Table 1. Key primers used for amplification of horse IL-12 p35 and p40 and IL-18 cDNAs. IL-12 primer sequences were derived from published human (Gubler et al. 1991), cattle (Zarlenga et al. 1995) and dog IL-12 sequences (D.J. Argyle, personal communi-

cation; Buttner et al. 1998) and from Rottman and co-workers (1996). IL-18 primers were derived from human IL-18 sequence (Ushio et al. 1996)

	N-terminal primers (5'-3')	C-terminal primers (5'-3')
p40	CAGAGCAAGATGTGTCACCAG AGATGCTGGCCAGTACACCT ATGTGTCACCAGTGGTTGGTC	CATACTGAGGTTAGAACC GGGAGAAGTAGGAATGTGGAG CAGGTGAGAATGGCTCAGAAC
p35	CAGTGCCGGCTCAGCATGTG GGATATGTGCCCCGCCGCGCG CA(A/C)GAATGAGAGTTGCCT	GAAGTATGCAGAGCTTGA GCTTTTATAGGAAGCATTTCAGATAGC CCGGTTCTTCAAGGGAGG
IL-18	GCAGGAATAAAGATGGCTGC [A] GACAATACGCTTTACTTTAT [C]	GCGTTTTGAACAGTGAACAT [B] GGCATGAAATTTTAATAGCTA [D]

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